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Molecular Characterization of Enrichment Cultures That Grow on Tetrachloroethene, 1,2 Dichloroethane and Ethylene Dibromide

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ABSTRACT

Halogenated aliphatic hydrocarbons are among the most common contaminants in soil and groundwater found at hazardous waste sites throughout the United States. Among them are tetrachloroethene (PCE), 1,2-dichloroethane (1,2-DCA) and ethylene dibromide (EDB). Organohalide respiration of each compound has been reported. However, considerably less information is known about EDB than PCE and 1,2-DCA, including the yield that occurs during growth with EDB as the sole TEA (Terminal electron acceptor). The main objective of this project was to determine which types of chlororespiring microbes predominate during growth of enrichment cultures when PCE, 1,2-DCA and EDB served as the TEA, and what their yield is. Based on previous studies, the genera of interest were *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium*.

The yield of *Dehalococcoides* during growth with EDB was $7.13 \pm 0.63 \times 10^6$ gene copies per µmol Br^-, based on qPCR (Quantitative Polymerase Chain Reaction) quantification of the 16S rRNA (Ribosomal Ribonucleic Acid) gene. This is similar to the yield reported by Eaddy (8), using a similar enrichment culture. No other reports of yields during respiration of EDB were found in the literature. *Dehalobacter* was also present in the EDB-grown enrichment culture, but at a concentration several orders of magnitude lower than *Dehalococcoides*. *Desulfitobacterium* was not detected, based on a lack of amplification of its 16S rRNA gene.

The yield of *Dehalococcoides* during growth with 1,2-DCA was $4.59 \pm 0.036 \times 10^7$ gene copies per µmol Cl^-. A similar yield was measured when the enrichment culture grown with EDB was switched to 1,2-DCA as the TEA, supporting the observation that *Dehalococcoides* were responsible for dehalogenation of both compounds in both enrichment cultures.

Dehalobacter and *Desulfitobacterium*, the two other genera known to respire 1,2-DCA, were present either at much lower concentrations than *Dehalococcoides*, or were not detected at all.

The yield for *Dehalococcoides* in the 1,2-DCA enrichment culture was similar in magnitude to the yield for PCE in this study ($2.11 \pm 0.082 \times 10^8$) and other pure and mixed cultures containing *Dehalococcoides* grown with chlorinated ethenes. It is not yet known why the yield for *Dehalococcoides* was significantly lower when grown with EDB. This is especially notable considering that, when EDB and 1,2-DCA are added to either enrichment culture at the same time, EDB is always used preferentially to 1,2-DCA (38). Overall, the results of this study contribute to a general understanding of organohalide respiration with chlorinated and brominated compounds.

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ABBREVIATIONS

1,2-DCA	1,2-dichloroethane
ABI	Applied Biosystems
cDCE	*cis*-1,2-dichloroethene
DDI	distilled, deionized
DNA	deoxyribonucleic acid
EDB	ethylene dibromide (1,2-dibromoethane)
FID	flame ionization detector
GC	gas chromatograph
PCE	tetrachloroethene (perchloroethylene)
qPCR	quantitative polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SRS	Savannah River Site
TCE	trichloroethene
TEA	terminal electron acceptor

CHAPTER ONE

1.0 INTRODUCTION

1.1 Overview

Halogenated organic solvents are among the most common groundwater contaminants found at industrial sites throughout the United States, including tetrachloroethene (PCE), 1,2-dichloroethane (DCA) and ethylene dibromide (EDB). PCE is a colorless liquid with a sweet odor, which is widely used for dry cleaning and degreasing. 1,2-DCA is a colorless liquid with a characteristic odor. It is mostly used in the production of vinyl chloride (VC), which is used as a monomer in the production of polyvinyl chloride. It is also used as a degreaser and paint remover. EDB is a colorless liquid with a sweet odor. It was historically used as an additive in leaded gasoline. Its function was to convert lead oxides that are produced during combustion, into lead halides, which are easily released with engine exhaust (21). It is now used as a fumigant and in the preparation of dyes and waxes. Both EDB and DCA have been classified by the Environmental Protection Agency as possible human carcinogens. The maximum contaminant level goal for drinking water has been set at zero for these compounds and the maximum contaminant level has been set at 5 µg/L for 1,2-DCA and PCE and 0.05 µg/L for EDB (34).

Although numerous subsurface remediation techniques are available, bioremediation is of particular interest since it is often effective and of low cost. Under the correct conditions, PCE and trichloroethene (TCE) undergo hydrogenolysis to ethene (12), while 1,2-DCA (15) and EDB (18, 20) undergo dihaloelimination to ethene. These processes occur when an electron donor provides the necessary reducing equivalents to replace the halogen atoms with hydrogen, in the presence of microbes capable of using the halogenated compounds as growth-linked terminal

electron acceptors (TEAs). The pathways for 1,2-DCA and EDB are shown in Figure 1.1 (figure replaced). Although ethene is formed predominantly via dihaloelimination, hydrogenolysis can result in small amounts of chloroethane (CA) and bromoethane (BA) from 1,2-DCA and EDB, respectively. Further reduction of these compounds to ethane is possible, but at a comparatively slow rate. Hydrogenolysis of CA and BA is not associated with organohalide respiration. Reduction of ethene to ethane is also possible; very little is known about the microbiology of this step. Another relatively minor transformation pathway involves dehydrohalogenation of 1,2-DCA to VC and EDB to vinyl bromide (VB), which is an abiotic process. VC and VB can undergo hydrogenolysis to ethene.

For reductive dechlorination of PCE and TCE, many types of microbes have been characterized that can respire these compounds to *cis*-1,2-dichloroethene (cDCE), including *Desulfitobacterium*, *Dehalobacter*, *Sulfurospirillum*, *Desulfuromonas*, and *Geobacter*. The only genus that is able to metabolically reduce cDCE and VC to ethene is *Dehalococcoides*. Some types of *Dehalococcoides* can also use PCE and TCE as terminal electron acceptors, and not all can use VC by organohalide respiration; their unifying characteristic is that all can reductively dechlorinate cDCE metabolically. Mixed cultures often contain various types of *Dehalococcoides* with different metabolic abilities, as well as other types of organohalide respiring microbes; this helps to ensure high rates of complete dechlorination to ethene. When sites lack the necessary microbes, one option is to add them along with an electron donor, a process referred to as bioaugmentation.

At the Savannah River Site (SRS), several groundwater plumes are contaminated with PCE and TCE. The absence of *Dehalococcoides* in these areas makes them potential candidates for bioaugmentation. One area at the site where natural attenuation is occurring is the C-area

burning rubble pit. Here, a plume of TCE discharges into the Twin Lakes wetland. Field and laboratory studies have shown that the TCE undergoes complete dechlorination to ethene and ethane within the wetland, which has very high levels of organic matters that serves as an inexhaustible supply of electron donor. SRS has sponsored several projects at Clemson University with the aim of developing a bioaugmentation culture using inoculum from the Twin Lakes wetland. The goal of this effort has been to produce an enrichment culture that can be used at other areas of the site that are not good candidates for natural attenuation or biostimulation, since the necessary microbes are absent. Through the sequential efforts of Bratt (3), Wood (37) and Eaddy (8), an enrichment culture has been developed and has been named "MicroCED." SRS and Clemson University hold a patent on this culture, which has been maintained for approximately four years with only PCE and TCE as electron acceptors and lactate as the electron donor.

Eaddy (8) evaluated various characteristics of MicroCED, including its range of electron acceptors for organohalide respiration. In addition to PCE and TCE, use of cDCE, 1,1-DCE, *trans*-1,2-DCE, VC, 1,2-DCA, EDB, and vinyl bromide was examined. All of these compounds were dehalogenated at high rates, after starting with only a 2% (v/v) inoculum of the PCE and TCE-grown MicroCED culture. Following repeated addition and consumption of the halogenated compounds, the final concentration of *Dehalococcoides* was measured and the net increase in *Dehalococcoides* was used to calculate a yield, in terms of 16S rRNA (Ribosomal Ribonucleic Acid) gene copies per µmol of chloride or bromide released. For the chlorinated compounds, yields ranged from 7.9E7 gene copies per µmol Cl^- for VC to 1.8E9 gene copies per µmol Cl^- for 1,2-DCA. The most puzzling result was the comparatively low yield for EDB:

6.8E5 gene copies per µmol Br^-. The rate of EDB dehalogenation was equal to or higher than for 1,2-DCA, yet the yield was far lower.

The EDB yield result from Eaddy (8) was the key motivation for the research reported in this thesis. Experiments were designed to provide more reliable measurements of the yield for 1,2-DCA and EDB; PCE was included as a reference compound. Using 2000 mL bottles to grow the cultures rather than 160 mL serum bottles, it was possible to take samples after increasing amounts of the compounds had been consumed, and thereby determine the yield of *Dehalococcoides* as a function of the amount of halide released. In addition, the possibility that *Dehalobacter* and/or *Desulfitobacterium* were responsible for EDB dehalogenation, rather than *Dehalococcoides,* was evaluated. Both types of microbes are known to use 1,2-DCA as a TEA, so this possibility was explored for EDB.

In the next section of the Introduction, the literature on bioaugmentation is reviewed for cultures that respire PCE and TCE. Since the method used to quantify increases in microbes based on their 16S rRNA gene is so central to the experiments performed in this project, section 1.3 provides an overview of the quantitative polymerase chain reaction (qPCR) method. The final section of the Introduction provides the specific research objectives.

1.2 Characteristics of Selected Cultures Used for Bioaugmentation

Microorganisms can use halogenated compounds such as PCE (15), 1,2-DCA (29) and EDB (28) as TEAs during organohalide respiration. Enrichment cultures and isolates of bacteria have been established for PCE (1, 8, 11), 1,2-DCA (6, 14-15, 26, 35) and EDB (8, 18, 20). In these enrichment cultures and pure isolates, three species of bacteria *Dehalococcoides* (8, 18,

20), *Desulfitobacterium* (6, 26, 35) and *Dehalobacter* (14-15, 33) are dominant in the biodegradation of halogenated ethenes and ethanes.

Numerous pilot-scale bioaugmentation trials are reported in the literature; several are summarized here. A pilot scale study was completed at Dover Air Force Base, Delaware, where a plume of TCE was partially reduced to cDCE, but no further (10). Complete reduction to ethene occurred only after bioaugmentation. Kelly Air Force Base, Texas faced a similar problem of stalling at cDCE from PCE. Here a different culture was used to produce complete reduction of PCE and cDCE to ethene (27). In many other studies bioaugmentation has been the preferred *in situ* bioremediation method (30). This has been justified by a comparative study between biostimulation (i.e., addition of electron donor to the contaminated site) and bioaugmentation, which showed that bioaugmentation took less than half the time of biostimulation (25).

In order to document bioaugmentation, ethene and other daughter products can be used to obtain a mass balance. However at many sites contaminated with these compounds the mass balance does not correlate with the degradation. This led to the development of molecular techniques which can provide a better understanding of the biodegradation process. Molecular techniques are being used more frequently for documenting biodegradation, including qPCR.

Rahm et al. (30) compared three molecular techniques: terminal restriction fragment length polymorphism, restriction fragment length polymorphism analysis with clone sequencing, and qPCR for finding the differences in the microbial communities at two sites (Idaho National Engineering and Environmental Laboratory; and Seal Beach, California). Both sites were initially subjected to biostimulation. At Seal Beach, PCE was degraded only to cDCE, while at the Idaho National Engineering and Environmental Laboratory, complete conversion of TCE to

ethene occurred after biostimulation with lactate. The Seal Beach site was then subjected to bioaugmentation, resulting in complete conversion to ethene. When qPCR targeting the 16S rRNA gene of *Dehalococcoides* was used, it revealed a significant population at Idaho National Engineering and Environmental Laboratory, but no detectable quantities at Seal Beach prior to bioaugmentation. Although the presence of *Dehalococcoides* was detected by qPCR at Idaho National Engineering and Environmental Laboratory site, it was not represented in either the terminal restriction fragment length polymorphism or the clone sequencing analysis. Also the results from qPCR correlated with the observed ethene production at both site. Based on these findings Rahm et al. (30) concluded that the qPCR method provided the most effective and direct prediction of dechlorination potential. qPCR is also more reproducible and accurate than other methods like optical density and cell count data (4).

Organohalide respiration of 1,2-DCA has been demonstrated with three genera: *Dehalococcoides* (29), *Dehalobacter* (15), and *Desulfitobacterium* (6, 26, 35). Although several strains of *Dehalococcoides* respire 1,2-DCA (e.g. strains 195 and BAV-1) (17), others do not (e.g., strains GT and FL2). Grostern and Edwards (15) evaluated the growth of *Dehalococcoides* and *Dehalobacter* in enrichment cultures prepared with samples from a former chlorinated solvent disposal facility in West Louisiana, in which 1,2-DCA and 1,1,2-trichloroethane were present. Using qPCR, they showed that *Dehalobacter* grew during dihaloelimination of 1,2-DCA to ethene and 1,1,2-trichloroethane to VC, while *Dehalococcoides* grew during hydrogenolysis of VC to ethene.

Desulfitobacterium dichloroeliminans strain DCA1 also reduces 1,2-DCA to ethene by organohalide respiration (6, 26, 35). In a lab scale study, De Wildeman et al. (6) showed that strain DCA1 was effective during bioaugmentation of groundwater samples contaminated with

40 mg/L of 1,2-DCA, using lactate as the electron donor. De Wildeman et al. (6) also reported a growth rate of more than 350 nmol Cl^- released/min/mg protein and there was no production of VC. Van Raemdonck et al. (35) focused on optimizing use of the qPCR technique to quantify strain DCA1 during organohalide respiration of 1,2-DCA. This technique proved effective in dectecting *Desulfitobacterium dichloroeliminans* strain DCA1 in groundwater samples from a bioaugmented monitoring well. Another study by the same group (35) evaluated the transport and activity of the *Desulfitobacterium dichloroeliminans* strain DCA1 in a bioaugmented industrial site in Belgium, using the qPCR technique developed by Van Raemdonck et al. (35). They observed a decrease in 1,2-DCA levels in the wells supplemented with sodium lactate, but to an extent lesser than the predicted values based on the lab scale study. They were able to show the transport of the *Desulfitobacterium dichloroeliminans* strain DCA1 from the injection well to a monitoring well. These results supported the use of bioaugmentation to remove 1,2-DCA from groundwater via reduction to ethene.

Compared to 1,2-DCA, relatively little is known about the microbes responsible for organohalide respiration of EDB. Only two studies were found that reported EDB use as a TEA by *Dehalococcoides* (18, 20). Dihaloelimination of EDB has not been reported for other strains, including *Dehalobacter* and *Desulfitobacterium*. Furthermore, no information was found regarding the yield during organohalide respiration of EDB, except for the unexpectedly low value for *Dehalococcoides* reported by Eaddy (8) for the MicroCED culture.

1.3 qPCR

qPCR assays are frequently used to detect and quantify bacteria based on the unique sequence of their 16S rRNA gene. qPCR is a molecular technique, which is being used widely in

many fields beyond environmental engineering, including medical diagnostics. This method is more accurate than other methods to quantify microbes, including cell counting, optical density, and terminal restriction fragment length polymorphism (30). qPCR (also known as real time PCR) is a molecular technique in which a PCR reaction is conducted to amplify and simultaneously quantify a targeted DNA molecule. A thermal cycler is used, which consists of cycles of repeated heating and cooling for DNA melting and enzymatic replication of the DNA. Short DNA fragments called primers, which contain sequences complementary to the target region, are used to selectively amplify the DNA. Taq polymerase is used as a heat stable enzyme; it uses single stranded DNA as a template and the primers to assemble new pieces of double stranded DNA. Before the thermal cycling process is started an initialization step is performed, during which the reaction mixture is heated to about 95^{o}C and then maintained for a period of time. Then the thermocycling process is started. A single cycle stage typically consists of the following three temperature steps:

1. Denaturation: This step causes the melting of the double stranded DNA into single stranded DNA. This is accomplished by the breaking of hydrogen bonds between the complementary nucleotide bases.
2. Annealing step: In this step the temperature is lowered to allow annealing of the primers to the single stranded DNA template. The Taq polymerase enzyme starts to bind to the primer-DNA template and produce new double stranded DNA.
3. Elongation step: In this step the Taq polymerase synthesizes a new strand of DNA, which is complementary to the single stranded DNA template by adding deoxyribonucleotide triphosphates. After each elongation step the amount of target DNA

is doubled. So after n cycles, the DNA synthesized will be 2^n times the initial amount of target DNA.

In a traditional PCR process, agarose gel electrophoresis is used to check for size separation of PCR products. With a newer qPCR technique, either a fluorescent dye (SYBR green) that binds to double stranded DNA is used or a dual-labeled probe (Taqman probe) is used. With the dual-labeled probe, two fluorophores are used; one is called a reporter and the other is called quencher. A fluorophore is a component of a molecule which causes a molecule to be fluorescent. It is a functional group in a molecule which will absorb energy of a specific wavelength and re-emit energy at a different (but equally specific) wavelength. When the reporter is excited by a laser, it absorbs that light and then emits a characteristic wavelength of light. As long as the quencher remains in close proximity it will absorb the light emitted by the reporter. As the dual-labeled primer binds to the target sequence and gets degraded by the 5' nuclease activity of the Taq polymerase, the quencher is moved away from the reporter. This results in an increase in fluorescence that is correlated with the specific amplification of the target sequence (Figure 1.2). During the PCR process an increase in the double stranded DNA produced causes an increase in fluorescence intensity, which is measured at each cycle in the thermal cycling process. The cycle time corresponding to an exponential increase in the fluorescence is recorded as the Ct value. The lesser the Ct value the higher the amount of DNA present in the sample. Standards are created with varying concentrations of plasmids containing the corresponding bacterial 16S rRNA gene fragments. Concentrations of gene copies in samples are determined using the standard Ct curve. This provides quantification in the form of an absolute number of copies of the DNA per milliliter of the culture (6).

In order to generate number of copies of the DNA per milliliter of the culture, the software (Sequence Detection System 2.2) makes a plot of ΔRn (fluorescence of the reporter dye divided by the fluorescence of a passive reference dye minus the baseline) versus the qPCR cycle. The software subdivides the plot in three phases: exponential, linear and plateau. The first phase is the exponential phase during which the PCR reaction is occurring at approximately 100% efficiency (doubling of product at end of each cycle). Over time the reaction slows down and is known as the linear phase. Ct is a cycle number corresponding to the intersection between the amplification curve and a threshold line. It is basically the relative measure of the concentration of the target in the qPCR reaction. A standard curve is generated for every set of qPCR reactions, by plotting the Ct value versus the natural log of the concentration (copies/µL) of the plasmid DNA. The equation of the curve along with the corresponding Ct value of the samples is then used to calculate the gene copy concentration in the samples.

1.4 Research Objectives

The main objective of this project was to determine which types of chlororespiring microbes predominate during growth of the MicroCED enrichment culture when PCE, 1,2-DCA and EDB serve as the TEA. To accomplish this, two variations of the MicroCED culture were developed; one was provided with 1,2-DCA as its only TEA; the other was provided with only EDB. These "Mother Cultures" were then used as a source of inoculum to determine which types of microbes grow during reductive dechlorination of PCE and dihaloelimination of 1,2-DCA and EDB. Based on previous studies, the genera of interest were *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium*. The specific objectives were:

1) To measure the yield of *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium* in an enrichment culture grown with 1,2-DCA as its TEA and seeded with the 1,2-DCA Mother Culture.

2) To measure the yield of *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium* in an enrichment culture grown with EDB as its TEA and seeded with the EDB Mother Culture.

3) To measure the yield of *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium* in an enrichment culture grown with 1,2-DCA as its TEA and seeded with the EDB Mother Culture;

4) To measure the yield of *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium* in an enrichment culture grown with EDB as its TEA and seeded with the 1,2-DCA Mother Culture; and

5) To measure the yield of *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium* in the MicroCED culture grown with PCE as its TEA and seeded with the MicroCED Culture.

The intent of "cross-inoculation" with 1,2-DCA and EDB (i.e., feeding 1,2-DCA to the EDB inoculum and EDB to the 1,2-DCA inoculum) was to further determine if the microbes responsible for dihaloelimination of 1,2-DCA are the same as those for EDB.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Chemicals and Media

Polymer grade ethene (99.9) was obtained from Matheson. PCE (99.9%) was obtained from Sigma-Aldrich. EDB (99%) was obtained from Acros Organics and 1,2-DCA (99%) from Mallinckrodt. Sodium lactate syrup (containing 58.8-61.2% sodium lactate; specific gravity = 1.31) was obtained from EM Science. SYBR green and Taqman PCR master mix were obtained from Applied Biosystems (ABI). The primers and probes were obtained from Integrated DNA Technologies. All other chemicals used were reagent grade, unless indicated otherwise.

The anaerobic mineral medium used was adapted from Edwards and Grbić-Galić (9). Appendix A provides a description of how the media was prepared.

2.2 Maintenance of Mother Cultures

Three enrichment cultures were used during this research and are identified as SRS, EDB and DCA. They are referred to as the Mother Cultures since they served as the sources of inoculum for the experiments.

The SRS Mother Culture uses PCE and TCE as its terminal electron acceptors via organohalide respiration to ethene, with lactate serving as the electron donor. It was started from microcosms that were constructed with soil and groundwater from the Twin Lakes area at the Savannah River Site (3). The enrichment culture was initially developed by Wood (37) and characterized by Eaddy (8). The DCA and EDB enrichment cultures were developed by Yu using the SRS culture as inoculum (38). They received DCA and EDB, respectively, as their

sole TEA and lactate as their electron donor; ethene was the predominant product, via dihaloelimination.

The Mother Cultures were grown in 2650 mL glass reagent bottles containing 1660 mL of liquid and 990 mL of headspace (or a headspace to liquid ratio of 0.6). The bottles were sealed with a Teflon-faced septum (35 mm) placed inside a plastic screw cap; 3 mm holes were drilled in the cap to provide access for sampling using a syringe. The septum was replaced periodically to prevent diffusive losses of the volatile compounds. The bottles were incubated quiescently, shielded from light, at room temperature, and with the liquid in contact with the septum to minimize the loss of volatile compounds.

The SRS Mother Culture received neat TCE (580 µmol/bottle, resulting in an aqueous phase concentration of 14.6 mg/L when taking partitioning to the headspace into account) and neat PCE (190 µmol/bottle, resulting in an aqueous phase concentration of 13.9 mg/L when taking partitioning to the headspace into account) in approximately two week intervals. The actual amounts added were determined gravimetrically by weighing the syringe with the compound before and after adding the compound. Lactate (1 mL of a 0.334 g/L solution of sodium lactate syrup) was added each time the enrichment bottles were sampled for headspace analysis and each time PCE and TCE were added. After two feeding cycles of PCE and TCE, 300 mL of culture was removed from the SRS enrichment culture and replaced with fresh media, before addition of PCE, TCE and lactate. Addition of fresh media provided the necessary nutrients and prevented accumulation of salts from neutralization of HCl with NaOH.

The DCA and EDB Mother Cultures were maintained in a similar manner. The DCA Mother Culture received neat 1,2-DCA (380 µmol/bottle, resulting in an aqueous phase concentration of 24.2 mg/L when taking partitioning to the headspace into account). The EDB

Mother Culture received neat EDB (180 µmol/bottle, resulting in an aqueous phase concentration of 15.0 when taking partitioning to the headspace into account). The actual amounts added were determined gravimetrically. The frequency of additions was weekly. Lactate (1.5 mL of a 0.334 g/L solution of sodium lactate syrup) was added each time the cultures were sampled for headspace analysis and each time EDB and DCA was added. After every fourth cycle of EDB and DCA addition and dehalogenation, 300 mL of culture was removed and replaced with fresh media.

For all of the Mother Cultures, pH was measured in 0.5 mL samples using a Corning 345 pH meter and VWR SympHony probe. The pH meter was calibrated using pH 4.0 and 7.0 buffer solutions before samples were analyzed. If the pH of the culture was below 6.6, 0.1 mL of 8 M NaOH was added. After equilibrating for 30 min on a stir plate, a new sample was removed and the pH was re-measured. This process was repeated until the culture pH was approximately 7.

2.3 Experimental Design and Protocol

Yields were measured for six experimental conditions, as summarized in Table 2.1 (Not addressed). For treatment #1, PCE was used as the TEA in order for growth of the SRS enrichment culture. This served as a positive control for the growth of *Dehalococcoides*, since Eaddy (8) had previously measured a yield for this culture. For treatment #2, the inoculum was the DCA Mother Culture and 1,2-DCA was provided as the TEA. For treatment #3, the inoculum was the EDB Mother Culture and EDB was provided as the TEA. For treatment #4, the inoculum was the DCA Mother Culture and EDB was provided as the TEA. For treatment #5, the inoculum was the EDB Mother Culture and 1,2-DCA was provided as the TEA. Treatment #6 consisted of water controls.

In order to provide sufficient sample to detect the presence of *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium*, the experiments were performed in custom-fabricated 2 L glass bottles; threaded glass tubes for installing Mininert valves were added by a glass blower (Figure 2.1). Treatments #1-5 were started with 1960 mL of minimal media and 40 mL of inoculum (2%). The media and culture were added to the bottles inside an anaerobic chamber. Once assembled, the bottles were removed from the chamber and the headspaces were purged (1 min) with a gas mixture (30% CO_2, 70% N_2), in order to equilibrate with bicarbonate in the medium and lower the pH to approximately 7; if the pH was more than 0.2 units away, HCl or NaOH was added for final adjustment. The initial amount of PCE added was based on the amounts used by Eaddy (8); since she used 160 mL serum bottles containing 100 mL of culture, the amounts were scaled to the larger bottles used in this study (Table 2.2). The amount of PCE added was gradually increased, to a maximum of about 570 µmol/bottle. After three cycles of PCE addition and at least 95% reduction to ethene, the first 200 mL sample was removed, leaving 1800 mL in the bottle. Samples were taken inside an anaerobic chamber (with an atmosphere of approximately 1.5% hydrogen and 98.5% nitrogen) by removing the screw cap, pouring the sample into a graduated cylinder, and immediately replacing the cap. At the end of each subsequent cycle of PCE addition and reduction to ethene, 200 mL of sample was removed, so that at the end of the seventh cycle, five samples of 200 mL each were available for analysis by qPCR, leaving 1000 mL of culture in the bottle. Table 2.2 also shows the calculated amount of chloride released for each cycle, along with the total chloride released, based on the expected amount of PCE reduced to ethene. Lactate was added each time the experimental bottles were sampled for headspace analysis and each time the TEAs were added (2 mL of a 0.334 g/mL

solution of 60% sodium lactate syrup). The total amount of lactate added was at least 100 times greater than the electron donor needed for stoichiometric dehalogenation of the PCE.

The experimental procedure for treatments that received 1,2-DCA (#2 and #5) and EDB (#3 and #4) are shown in Tables 2.3 and 2.4, respectively. The same approach that was used for PCE was used for these compounds, i.e., three cycles of dehalogenation before the first 200 mL sample was taken for qPCR analysis, followed by removal of 200 mL samples after each subsequent cycle until seven cycles of addition and dehalogenation were completed. As with PCE, the expected amounts of chloride and bromide released were calculated based on the respective amounts of 1,2-DCA and EDB consumed.

An alternative approach to this process was considered, i.e., replacement of the 200 mL sample with fresh media. However, because of the dilution effect this would have caused, this was deemed to be less desirable than having the liquid volume decrease over time.

The initial concentration of *Dehalococcoides, Dehalobacter* and *Desulfitobacterium* was determined by measuring levels in the Mother Cultures and dividing by the dilution factor (50-fold). Selection of a 200 mL sample size was based on preliminary measurements made with the Mother Cultures and estimating the minimum level of detection by qPCR.

In addition to the live treatments, one set of water controls (WCs) was prepared to evaluate the extent of abiotic losses via diffusion. The WCs contained distilled, deionized (DDI) water + PCE, 1,2-DCA, EDB, and ethene. Ethene was added to the controls using a 10 mL Pressure-Lok® gas syringe. The WCs were prepared and incubated on the bench top with room air present in the headspace.

All of the experimental bottles were incubated on magnetic stir plates at room temperature (21-24^{o}C). A thin piece of Styrofoam (0.1 mm) was placed between the stirrer and

the bottle, to reduce heat transfer from the stir plates to the cultures. After each addition of neat PCE, 1,2-DCA, or EDB, the bottles were allowed to equilibrate for at least one hour before a headspace sample was removed for analysis by gas chromatography (see below). pH was measured and adjusted in the experimental bottles in the same manner described above for the Mother Cultures.

2.4 Analysis of Volatile Organic Compounds

Volatile organic compounds were monitored by analysis of headspace samples (0.5 mL) on a Hewlett Packard Series II 5890 Gas Chromatograph (GC) equipped with a packed column (1% SP-1000 on 60/80 Carbopack-B; Supelco, Inc.) and flame ionization detector (FID), as previously described (13). The carrier gas used was nitrogen. The total mass of a VOC in the bottles was determined by response factors, based on the GC response to injection of a headspace sample (i.e., total mass per bottle per peak area unit from a 0.5 mL headspace sample). Headspace samples from the Mother Culture bottles were removed via the septum in the screw cap that sealed the bottles; samples from the experimental bottles (Figure 2.1) were removed via the Mininert valve.

For the SRS Mother Culture, the response factors measured by Wood (37) for 160 mL serum bottles were used for PCE, TCE, cDCE, VC, ethene and methane. The same response factors were applicable to the SRS Mother Culture since the ratio of headspace to liquid was the same in both types of bottles (i.e., 0.6). To obtain the total mass of VOCs in the SRS Mother Culture, the response factors from Wood (37) were multiplied by the ratio of the total volume of the bottles (i.e., 2650 mL/160 mL) (37). For the DCA and EDB Mother Cultures, the same

approach was used, based on response factors determined by Eaddy (8) for 1,2-DCA, chloroethane, VC, EDB, bromoethane, vinyl bromide, ethene, ethane and methane.

Response factors for the custom fabricated 2 L experimental bottles (Figure 2.1) were measured for PCE, 1,2-DCA, EDB and ethene. Since the liquid and headspace volumes changed over time (i.e., from 2000 mL at the start to 1000 mL at the end, in 200 mL increments), it was necessary to determine multiple response factors. A gravimetrically determined amount of PCE, 1,2-DCA, and EDB was added along with a known amount of ethene (based on the volume added at a measured temperature and pressure), to four bottles containing DDI water. Headspace samples were analyzed on the GC after allowing the headspace and liquid phases to equilibrate for 1 hr. This process was repeated for liquid volumes of 2000, 1800, 1600, and 1400 mL of DDI water; standard curves are shown in Appendix B. For the 1200 mL condition, responses factors were estimated by linear extrapolation of the results for the experimentally determined response factors at the higher liquid volumes (Figure B-5 and Table B.8). It was not necessary to determine response factors at a liquid volume of 1000 mL, since operation of the bottles ceased after removing the final 200 mL sample.

Given the history of Mother Cultures, it was anticipated that daughter products other than ethene would be minor. Therefore, rather than experimentally determining response factors for TCE, cDCE, VC, chloroethane, bromoethane, and vinyl bromide, response factors for these compounds were estimated based on proportional responses to compounds with similar response factors in serum bottles (Appendix B, Tables B.4-B.8). The results confirmed that these daughter products were either transient or remained minor throughout the experimental period.

The GC response to a headspace sample was calibrated to give the total mass of the compound (M) in that bottle. Assuming that the headspace and aqueous phases were in

equilibrium, the total mass present was converted to an aqueous phase concentration using the following formula.

$$C_l = \frac{M}{V_l + H_c V_g} \tag{2-1}$$

where C_l = concentration in the aqueous phase (µM); M = total mass present (µmol/bottle); V_l = volume of the liquid in the bottle (L); V_g = volume of the headspace in the bottle (L); and H_c = Henry's constant (dimensionless) at 23°C (4).

2.5 DNA Extraction

Genomic DNA was extracted from liquid samples by centrifuging 200 mL at 4500x*g* and 4°C using a Sorvall Evolution RC centrifuge (15). The 200 mL culture volume was decided based on initial qPCR tests with the three mother bottles, which showed consisted amplification with DNA extracted from 200 mL culture volume. The pellet formed was transferred into a Bead tube (MoBio's UltraClean soil microbial DNA isolation kit) and extracted using MoBio's UltraClean soil microbial DNA isolation kit, according to the manufacturer's protocol, with one exception: the final volume of Tris eluent used was 60 µL.

Plasmids with the 16s rRNA gene of *Dehalococcoides* and *Dehalobacter* were extracted by first growing them overnight in the incubator on culture plates with LB media and agar. A colony was selected and grown again in 20 mL of LB media in a 125 mL Erlenmeyer flask. Following growth, the culture was centrifuged (5400x*g*, 10 min, 4°C). DNA was extracted from a white pellet that formed using the QIAprep Spin Miniprep Kit, in accordance with the manufacturer's protocol (17). Aseptic techniques were used during all stages of the extraction. Extracted DNA was stored at -20°C.

All the glassware, pipette tips, centrifuge tubes and DDI water used for DNA extraction and qPCR (see below) was autoclaved at 121^{o}C for 40 min with a 3 min purge time, two pulses, and 10 min of drying time.

2.6 Quantitative Polymerase Chain Reaction

The concentration of *Dehalococcoides*, *Dehalobacter* and *Desulfitobacterium* species was determined in the extracted DNA using qPCR. The qPCR reactions were performed in an ABI 7900HT machine. SYBR green (15) and Taqman PCR master mixes from ABI were used to prepare the qPCR reaction mixtures. The SYBR green master mix contains the necessary Taq polymerase enzyme, buffers, passive reference and deoxyribonucleotide triphosphates, and SYBR green dye. The Taqman PCR master mix contains the same reagents minus the SYBR green dye. Primers and probes specific to each of the species listed in Table 2.5 were used. To amplify the *Dehalococcoides* 16S rRNA gene, the Taqman probe was used (17); to amplify *Dehalobacter* and *Desulfitobacterium*, SYBR green dye was used for qPCR (33).

Each time qPCR was performed, a set of standards was included. The standards consisted of the target species 16S rRNA gene in a plasmid. *Dehalococcoides* 16S rRNA gene in a plasmid was provided in DNA form by Dr. Christopher Bagwell (Savannah River National Laboratory) along with information concerning its concentration in copies/µL. *Dehalobacter* 16S rRNA gene in a plasmid was provided by Dr. Frank Löffler (University of Tennessee, Knoxville) along with information about the plasmid size (562 base pairs). Standards for *Desulfitobacterium* qPCR could not be obtained. Nevertheless, qPCR was performed to determine if there was any amplification of DNA; since there was not, no further attempts were made to obtain standards.

For *Dehalococcoides*, the plasmids containing the 16S rRNA gene were used directly as a standard. For *Dehalobacter*, it was necessary to extract the plasmid containing its 16S rRNA gene from the host cell (*E. coli*) using a QIAprep Spin Miniprep kit, according to the manufacturer's protocol. The extract was analyzed in a UV spectrophotometer in order to determine the concentration of DNA (23). DNA absorbs UV light at 260 nm with an average extinction coefficient of 0.020 $(\mu g/mL)^{-1}$ cm^{-1}. After estimating the concentration of plasmid DNA, the concentration of *Dehalobacter* in terms of gene copies/µL was calculated using the following formula (17):

$$(\text{gene copies}/\mu L) = \left(\text{DNA concentration}\left[\frac{ng}{\mu L}\right]\right)\left(\frac{1\ g}{1000^3\ ng}\right)\left(\frac{1\ mol\ bp\ DNA}{660\ g\ DNA}\right)\left(\frac{6.023 \times 10^{23}\ bp}{mol\ bp}\right)\left(\frac{1\ copy}{plamsid\ size\ bp}\right) \quad (2\text{-}2)$$

Based on this estimate, a serial dilution of the plasmid DNA was used to generate a standard curve for qPCR analysis. SYBR green was used as a fluorescent dye for *Dehalobacter* and *Desulfitobacterium* qPCR (33), which intercalated with the double-stranded DNA formed during the qPCR process. Taqman probes were used for *Dehalococcoides* qPCR (6). In Taqman based qPCR, the 5'-3' nuclease activity of Taq polymerase enzyme is used to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorescence of a fluorophore. Hence, fluorescence detected in the ABI 7900HT is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

The qPCR reaction mixture was pipetted into an ABI 96 well clear plate and then loaded into ABI 7900HT. Each *Dehalococcoides* 16S rRNA gene based qPCR reaction mixture (25 µL total volume) consisted of 12.5 µL of Taqman universal PCR master mix, 1 µL each of DHC 1200f, DHC 1271R and DHC 1240 Probe (all three 9 µM), 3.5 µL DDI water and 6 µL of

template DNA (17). Each *Dehalobacter* and *Desulfitobacterium* qPCR reaction mixture (50 µL total volume) consisted of 24 µL of SYBR green PCR master mix, 1 µL each of DHB 441f and DHB 645R for *Dehalobacter* and DSB 406F and DSB 619R for *Desulfitobacterium* (both at 0.5 µM), 19 µL DDI water and 4 µL of template DNA (33). The primers and probe (if used) were diluted to the required concentration using sterilized DDI water and then the total quantity required for a set of qPCR reactions was mixed in a 1.5 mL amber centrifuge tube along with the PCR master mix. The template DNA was first pipetted into the 96 well ABI plate and then the primer, probe and PCR master mix is pipetted into the reaction wells. A set of negative template controls (DDI water used instead of DNA) was included in every set of qPCR reactions, in order to determine the background fluorescence. This also helped to confirm that there was no contamination in the qPCR reagents. A low light environment was maintained in order to prevent deterioration of the light sensitive PCR master mix and Taqman probes. After all the PCR reaction mixtures were pipetted into the 96 well plate. The plate was sealed with an optical film cover from ABI and then vortex for 10 seconds and then centrifuged for 1 min. The centrifuge used for the 96 well plates was made by fixing two of the 96 well adaptors in a salad spinner, which was spun by a hand cranked lever (http://bitesizebio.com/2010/03/12/how-to-build-a-plate-centrifuge-for-25/). Then the 96 well plate was loaded into the ABI 7900HT machine.

The thermocycling program for *Dehalococcoides* 16S rRNA gene targeted qPCR was: 2 min at 50^{o}C and 10 min at 95^{o}C, followed by 40 cycles of 15 s at 95^{o}C and 1 min at 58^{o}C (17). For *Dehalobacter* and *Desulfitobacterium*, the program used was: 15 min 94^{o}C initial denaturation, followed by 50 cycles of 30 s at 94^{o}C, 20 s at 58^{o}C and 30 s at 72^{o}C (15). The ABI 7900HT system uses sequence detection software to set various parameters, including the thermo-cycling program and type of detector. It also generates the Ct (threshold cycle) values

for the standards and the samples. The Ct values were then plotted against the natural log of the concentrations (copies/µL) of the serially diluted plasmid DNA. The equation for the tread line, the volume of culture used to extract DNA (200 mL), and the volume of DNA extract used, was used to calculate the quantity (copies/mL) in the samples. Aseptic techniques were used during all stages of qPCR.

The complete protocol for the qPCR method is described in Appendix C. Standard curves are provided in Appendix D.

CHAPTER THREE

3.0 RESULTS

3.1 SRS, DCA and EDB Mother Cultures

The performance of the SRS Mother Culture during the period of time it was used for this project is shown in Figure 3.1. Five additions of PCE and TCE were dechlorinated to ethene, with only transient appearance of chlorinated daughter products. The 200 µmol of PCE and 575 µmol of TCE added each time correspond to aqueous phase concentrations of 14.7 and 38.6 mg/L, respectively (using equation 2.1). The amount of ethene that formed was 87% (molar basis) of the PCE and TCE consumed (Table 3.1). An electron balance over the 84 days shown indicates that 0.007% of the electron equivalents (eeq) of lactate added was recovered as methane, while 0.14% was used for reductive dechlorination. The two times when culture was removed and fresh media were added are also shown on Figure 3.1.

The performance of the DCA Mother Culture during the period of time it was used for this project is shown in Figure 3.2. Six additions of 1,2-DCA were dechlorinated to ethene, with only minor amounts of VC. The 360 µmol of 1,2-DCA added each time corresponds to an aqueous phase concentration of 23 mg/L (using equation 2.1). The amount of ethene that formed was 92% (molar basis) of the 1,2-DCA consumed (Table 3.1). An electron balance over the 47 days shown indicates that 0.017% of the electron equivalents (eeq) of lactate added was recovered as methane, while 0.017% was used for reductive dechlorination.

The performance of the EDB Mother Culture during the period of time it was used for this project is shown in Figure 3.3 (right hand axis label changed from , to and). Eight additions of EDB were dechlorinated to ethene, with only minor amounts of VC. The 230 µmol of EDB added each time corresponds to an aqueous phase concentration of 28.8 mg/L (using equation

2.1). The amount of ethene that formed was 89% (molar basis) of the EDB consumed (Table 3.1). An electron balance over the 56 days shown indicates that 0.011% of the electron equivalents (eeq) of lactate added was recovered as methane, while 0.031% was used for reductive dechlorination. EDB was considerably more inhibitory to methanogenesis than 1,2-DCA.

3.2 Experimental Bottles

The performance of the duplicate experimental bottles that were inoculated with the SRS Mother Culture and provided with PCE (treatment #1 in Table 2.1) is shown in Figure 3.4. During the first two cycles of PCE addition and consumption, there was a transient accumulation of chlorinated daughter products. Thereafter, most all of the PCE was reduced directly to ethene. This is consistent with the behavior of the SRS Mother Culture, which was also provided with PCE and lactate (Figure 3.1). The highest amount of PCE added, 268 µmol/bottle, corresponds to aqueous phase concentrations of 21.7 mg/L when the liquid volume was 1600 mL (using equation 2.1). The total amount of ethene formed was 97% (molar basis) of the PCE consumed (Table 3.1). An electron balance over the 84 days shown indicates that 1.45% of the electron equivalents (eeq) of lactate added was recovered as methane (not shown in Figure 3.4), while 0.6% (addressed in discussions) was used for reductive dechlorination (Table 3.2). The five occasions when 200 mL samples were removed for qPCR analysis are shown in Figure 3.4; the two other samples taken were at time zero and at the end of the incubation period.

The performance of the duplicate experimental bottles that were inoculated with the DCA Mother Culture and provided with 1,2-DCA (treatment #2 in Table 2.1) is shown in Figure 3.5. Following a lag of approximately one month, a high rate of 1,2-DCA consumption was

underway. At all times, ethene was the only significant daughter product; CA and VC were minor. This is consistent with the behavior of the DCA Mother Culture (Figure 3.2). The highest amount of 1,2-DCA added, 527 µmol/bottle, corresponds to aqueous phase concentrations of 41.4 mg/L when the liquid volume was 1200 mL (using equation 2.1). The total amount of ethene formed was 95% (molar basis) of the 1,2-DCA consumed (Table 3.1). An electron balance over the 120 days of incubation indicates that 0.64% of the electron equivalents (eeq) of lactate added was recovered as methane (not shown in Figure 3.5), while 0.4% was used for reductive dechlorination (Table 3.2). The five occasions when 200 mL samples were removed for qPCR analysis are shown in Figure 3.5; the two other samples taken for qPCR were at time zero and at the end of the incubation period.

The performance of the duplicate experimental bottles that were inoculated with the EDB Mother Culture and provided with EDB (treatment #3 in Table 2.1) is shown in Figure 3.6. As with 1,2-DCA, a high rate of EDB consumption started following a lag period of approximately one month. At all times, ethene was the only significant daughter product; BA and VB were minor. This is consistent with the behavior of the EDB Mother Culture (Figure 3.3). The highest amount of EDB added, 523 µmol/bottle, corresponds to aqueous phase concentrations of 79.7 mg/L when the liquid volume was 1200 mL (using equation 2.1). The total amount of ethene formed was 91% (molar basis) of the EDB consumed (Table 3.1). An electron balance over the 120 days of incubation indicates that 0.01% of the electron equivalents (eeq) of lactate added was recovered as methane (not shown in Figure 3.5), while 0.4% was used for reductive dechlorination (Table 3.2). The five occasions when 200 mL samples were removed for qPCR analysis are shown in Figure 3.6; the two other samples taken for qPCR were at time zero and at the end of the incubation period.

The performance of the duplicate experimental bottles that were inoculated with the DCA Mother Culture and provided with EDB (treatment #4 in Table 2.1) is shown in Figure 3.7. It took longer before a consistently high rate of EDB consumption was underway in comparison to the treatment inoculated with the EDB Mother Culture (Figure 3.6); nevertheless, the switch to EDB as the TEA did occur. At all times, ethene was the only significant daughter product; BA and VB were minor. This is consistent with the behavior of the EDB Mother Culture (Figure 3.3) and treatment #3 (Figure 3.6). The highest amount of EDB added, 626 µmol/bottle, corresponds to aqueous phase concentrations of 95.4 mg/L when the liquid volume was 1200 mL (using equation 2.1). The total amount of ethene formed was 98% (molar basis) of the EDB consumed (Table 3.1). An electron balance over the 100 days of incubation indicates that 0.11% of the electron equivalents (eeq) of lactate added was recovered as methane (not shown in Figure 3.7), while 0.5% was used for reductive dechlorination (Table 3.2) (Order and name changed). The five occasions when 200 mL samples were removed for qPCR analysis are shown in Figure 3.7; the two other samples taken for qPCR were at time zero and at the end of the incubation period.

The performance of the duplicate experimental bottles that were inoculated with the EDB Mother Culture and provided with 1,2-DCA (treatment #5 in Table 2.1) is shown in Figure 3.8. It took longer before a consistently high rate of 1,2-DCA consumption was underway in comparison to the treatment inoculated with the DCA Mother Culture (Figure 3.5); nevertheless, the switch to 1,2-DCA as the TEA did occur. At all times, ethene was the only significant daughter product; VC and CA were minor. This is consistent with the behavior of the DCA Mother Culture (Figure 3.2) and treatment #2 (Figure 3.5). The highest amount of 1,2-DCA added, 607 µmol/bottle, corresponds to aqueous phase concentrations of 47.6 mg/L when the

liquid volume was 1200 mL (using equation 2.1). The total amount of ethene formed was 94% (molar basis) of the 1,2-DCA consumed (Table 3.1). An electron balance over the 140 days of incubation indicates that 2.24% of the electron equivalents (eeq) of lactate added was recovered as methane (not shown in Figure 3.7), while 0.4% was used for reductive dechlorination (Table 3.2). The five occasions when 200 mL samples were removed for qPCR analysis are shown in Figure 3.8; the two other samples taken for qPCR were at time zero and at the end of the incubation period.

Results for the duplicate water controls with PCE, 1,2-DCA, EDB and ethene (treatment #6 in Table 2.1) are shown in Figure 3.9. Only minor losses occurred over the 150 days of incubation, which was longer than any of the live treatments shown in Figures 3.4-3.8.

3.3 qPCR and Yield Results for *Dehalococcoides*

The seven samples taken from the duplicate experimental bottles were evaluated by qPCR for *Dehalococcoides*. Results are plotted in Figures 3.10-3.15 for the five live treatments (Table 2.1, Figures 3.4-3.8). The number of gene copies per mL is plotted against the cumulative amount of chloride or bromide released per mL of culture. Chloride and bromide release were calculated based on the amount of PCE, 1,2-DCA, and EDB consumed. The slope of the best fit line provides the yield, in gene copies per µmol of Cl^- or Br^-. For four of the five treatments (#1, 2, 3 and 5; Table 2.1), the coefficient of determination for the best fit line ranged from 83.8% to 96.8%, indicating a strong relationship between the increase in *Dehalococcoides* and the amount of dehalogenation of PCE, 1,2-DCA and EDB. The exception was treatment #4, which was inoculated with the DCA Mother Culture and received EDB as the TEA (Figure 3.13) (Figures 3.13 and 3.12 swapped). Although the slope of best fit line for data from both bottles is

statistically significant, there were considerable differences in the trends for the two bottles, as well as considerable variability among the replicate samples.

The yield results for *Dehalococcoides* are summarized in Figure 3.15 and reveal the following trends: $Y_{PCE} > Y_{1,2\text{-DCA}} > Y_{EDB}$. The yield for 1,2-DCA was about 50% lower when the inoculum was the EDB Mother versus the DCA Mother; nevertheless, even the lower yield for treatment #5 was significantly above the yield for EDB (treatment #3). The yield for treatment #4 (EDB with the 1,2-DCA Mother Culture as the inoculum) is not shown because of the poor fit of the data for gene copies and bromide release (Figure 3.13).

3.4 qPCR Results for *Dehalobacter* and *Desulfitobacterium*

qPCR analysis of the three Mother Cultures indicated the gene copy concentrations for *Dehalobacter* were close to or below the method detection limit of 10^3 copies per mL (Figure 3.16). In contrast, the concentrations for *Dehalococcoides* were readily determined. The concentration of *Dehalococcoides* in the EDB Mother Culture (2.86E+06 copies/mL) was considerably lower than in the other Mother Cultures, but well above the method detection limit of 10^3 gene copies per mL.

The concentration of *Dehalococcoides* and *Dehalobacter* from each of the experimental treatments is also plotted in Figure 3.16. These represent results for the final samples that were analyzed from duplicate bottles, at the end of the incubation period (Figures 3.4-3.8). As in the Mother Cultures, *Dehalococcoides* were readily quantified by qPCR while *Dehalobacter* were close to or below the detection limit. Since the results for *Dehalobacter* were so low relative to *Dehalococcoides* and therefore not show up well in Figure 3.16, the *Dehalobacter* concentrations above the detection limit are shown in Table 3.3. The final concentrations of *Dehalococcoides*

coincided with the yields shown in Figure 3.15, i.e., the concentration in the treatment with PCE (#1) was higher than in the treatments with 1,2-DCA (#2 and 5), which in turn were higher than in the treatment with EDB (#3). Treatment #4 is also shown (EDB, inoculated with the DCA Mother Culture), although the uncertainty associated with this result is high.

Because *Dehalobacter* concentrations in the final samples from the experimental bottles were either close to or below the detection levels, the decision was made not to analyze the earlier samples (i.e., with less PCE, 1,2-DCA and EDB consumed) by qPCR. It is evident from these results that *Dehalobacter* did not play a role in dehalogenation of PCE, 1,2-DCA, or EDB in the experimental treatments, or in the Mother Cultures. *Dehalobacter* levels were notable only in treatment #3, i.e., fed EDB and inoculated with the EDB Mother Culture. In this case, the final *Dehalobacter* concentration was approximately one order of magnitude lower than the final *Dehalococcoides* concentration (Figure 3.16).

Although a standard curve was not available for qPCR analysis of *Desulfitobacterium*, samples from the Mother Cultures and the final sample from the experimental bottles were subjected to qPCR using primers for this genus. However, none of the samples amplified, indicating that, like *Dehalobacter*, *Desulfitobacterium* were either absent or present in very low numbers. Dehalogenation of PCE, 1,2-DCA, and EDB in the Mother Cultures and the experimental treatments was limited to the growth of *Dehalococcoides*, not *Dehalobacter* or *Desulfitobacterium*.

4.0 DISCUSSION

The results of this study demonstrated that, in the enrichment cultures evaluated, *Dehalococcoides* are the microbes responsible for organohalide respiration of PCE, 1,2-DCA and EDB. This did not come as a surprise for PCE and 1,2-DCA; the results for this study were in agreement with those of Eaddy (8), who also measured yields for *Dehalococcoides* by the SRS MicroCED culture for the same compounds. However, the focus of this study was on the microbes responsible for EDB dehalogenation, given the unexpectedly low yield reported by Eaddy (8). The results confirmed the low yield observed by Eaddy (8) for EDB, when the EDB Mother Culture served as the inoculum source. This result was supported by qPCR analysis of the Mother Cultures; *Dehalococcoides* are present in the EDB Mother Culture at considerably lower levels than in the DCA and SRS Mother Cultures (Figure 3.16). The results from this study and Eaddy (8) are the only reports in the literature for yields on EDB by *Dehalococcoides*.

Methanogenesis was inhibited in the experimental bottles because of the toxicity of the TEA's added to the methanogenic bacteria. The lactate which was not use for either dehalogenation or for methanogenesis could have been fermented to organic acids, such as acetate and propionate.

Although the results clearly establish the lower growth yield for EDB in comparison to 1,2-DCA, it remains unclear why this is the case. Thermodynamics do not offer an explanation. Dihaloelimination of EDB is slightly more favorable than dihaloelimination of EDB (21); under standard conditions at pH 7, reduction of EDB with H_2 to ethene has a $\Delta G^{o'}$ of -195 kJ/mol, versus -188 kJ/mol for the same reaction with 1,2-DCA. The lower yield for EDB may be a consequence of significantly greater toxicity to *Dehalococcoides* compared to 1,2-DCA or the concentration of EDB used may be too high. The lower yield may also be due to the specificity

of the primers used and that there may be a unique *Dehalococcoides* present in the treatments fed with EDB, that may not have been amplified. With at least one other compound, the yield for the brominated version of a compound was lower than the chlorinated version. Scholtz et. al. (32) reported that the yield for a methylotrophic (microorganisms that can use reduced one-carbon compounds, such as methanol or methane as the carbon source for growth) bacterium grown on dibromomethane was approximately 26% lower than the yield on dichloromethane. In this case, the compounds were used as sole carbon and energy sources under aerobic conditions.

The greater toxicity of brominated versus chlorinated compounds to strictly anaerobic microbes has been demonstrated. Gunsalus et. al. (16) compared the inhibitory effects of bromoethanesulfonate and chloroethanesulfonate on the reduction of methyl-coenzyme M, which plays a key role in the pathway for methanogenesis. Coenzyme M is involved in the final steps of methane biosynthesis (36). Gunsalus et. al. (16) reported 50% inhibition of coenzyme M at a concentration of 7.9E-6 M for bromoethanesulfonate versus 7.5E-5 M for chloroethanesulfonate (reference deleted).

Nevertheless, some brominated compounds are not worse substrates than the chlorinated version. Eaddy (8) found that the SRS MicroCED enrichment culture had a higher yield during organohalide respiration of VB compared to VC. Other brominated organic compounds of interest in the environment include flame retardants: polybrominated diphenyl ethers, which also undergo reductive debromination by *Dehalococcoides* (24). Thus, additional research is needed to understand why the yields for *Dehalococcoides* during organohalide respiration of EDB are so much lower than for 1,2-DCA.

Table 4.1 compares the yields for *Dehalococcoides* from this study with others, for PCE and 1,2-DCA (no previous data is available for EDB). The yield for the DCA Mother Culture

and the EDB Mother Culture grown on 1,2-DCA (treatments #2 and 5, respectively) were lower than for KB-1 (7) by 3- to 6-fold. Considering the variability in qPCR procedures, these results are reasonably similar. The yield for *Dehalococcoides* in the SRS enrichment culture was also about 3-fold lower than reported for a similar type of culture. Table 4.1 also shows yields for *Dehalococcoides* growing on TCE, cDCE and VC. In general, the values are in the range of 10^7 to 10^8 gene copies per µmol. This contrasts sharply with the yield in this study for EDB, which was in the range of 10^6 gene copies per µmol.

Having determined yields for EDB when grown with the EDB Mother Culture (treatment #3) and for 1,2-DCA when grown with the DCA Mother Culture (treatment #2), it is possible to understand why a yield was not determined for treatment #4, i.e., growth on EDB using the DCA Mother Culture as inoculum. Using the DCA Mother culture as inoculum, the initial concentration of *Dehalococcoides* was much higher than when the EDB Mother Culture was the inoculum. As a consequence, it was more difficult to detect an increase above the initial *Dehalococcoides* concentration, given the much lower yield on EDB. This also helps to explain the longer lag period prior to the onset of 1,2-DCA dechlorination in treatment #5 versus #2, i.e., since treatment #2 had a higher initial concentration of *Dehalococcoides* (coming from the DCA Mother Culture) compared to #5 (coming from the EDB Mother Culture).

Although *Dehalobacter* and *Desulfitobacterium* played little or no role in organohalide respiration of 1,2-DCA and EDB in the cultures used in this study, their potential role in EDB dehalogenation should be evaluated further. This is especially the case for microbes such as *Desulfitobacterium dichloroeliminans* strain DCA1, which uses 1,2-DCA as a TEA (6). No studies were found that evaluated the ability of this microbe to grow with EDB. Although EDB is not the most prevalent contaminant at hazardous wastes, interest in its biodegradation is likely

to continue due to its high level of toxicity to human, as reflected in its 100-fold lower maximum contaminant level compared to 1,2-DCA, PCE, and TCE.

CHAPTER FIVE

5.0 CONCLUSIONS

Based on the results of this study, the following conclusions were reached:

- An enrichment culture was grown via organohalide respiration of EDB and lactate as the electron donor. The yield of *Dehalococcoides* during growth with EDB was 7.13±0.63 × 10^6 gene copies per µmol Br^-, based on qPCR quantification of the 16S rRNA gene. This is similar to the yield reported by Eaddy (8), using a similar enrichment culture. No other reports of yields during respiration of EDB were found in the literature. *Dehalobacter* was also present in the EDB-grown enrichment culture, but at a concentration several orders of magnitude lower than *Dehalococcoides*. *Desulfitobacterium* was not detected, based on a lack of amplification of its 16S rRNA gene.
- An enrichment culture was grown via organohalide respiration of 1,2-DCA and lactate as the electron donor. The yield of *Dehalococcoides* during growth with 1,2-DCA was 4.59±0.036 × 10^7 gene copies per µmol Cl^-. A similar yield was measured when the enrichment culture grown with EDB was switched to 1,2-DCA as the TEA, supporting the observation that *Dehalococcoides* were responsible for dehalogenation of both compounds in both enrichment cultures. *Dehalobacter* and *Desulfitobacterium*, the two other genera known to respire 1,2-DCA, were present either at much lower concentrations than *Dehalococcoides*, or were not detected at all.
- The yield for *Dehalococcoides* in the 1,2-DCA enrichment culture was similar in magnitude to the yield for PCE in this study (2.11±0.082 × 10^8) and other pure and mixed cultures containing *Dehalococcoides* grown with chlorinated ethenes. It is not yet known why the yield for *Dehalococcoides* was significantly lower when grown with EDB. This

is especially notable considering that, when EDB and 1,2-DCA are added to either enrichment culture at the same time, EDB is always used preferentially to 1,2-DCA (38). Overall, the results of this study contribute to a general understanding of organohalide respiration with chlorinated and brominated compounds.

TABLES

Table 2.1 Experimental design.

Treatment No.	Inoculum (v/v)	Electron acceptor	No. of Bottles
1	SRS Mother Culture (2%)	PCE	2
2	1,2-DCA Mother Culture (2%)	1,2-DCA	2
3	EDB Mother Culture (2%)	EDB	2
4	1,2-DCA Mother Culture (2%)	EDB	2
5	EDB Mother Culture (2%)	1,2-DCA	2
6	none	Water controls	2

Table 2.2 Experimental procedure for the treatment receiving PCE as TEA.

			PCE Added			Cl^- Present		
Dose	Operating point[a]	Volume (mL)	µmol/100 mL[b]	µmol/bottle[c]	µL (neat)[d]	µmol/bottle[e]	µmol/ mL[f]	Total (µmol/mL)[g]
1	start	2000	4.9	98.0	10.0		0.0	0.0
	end	2000				392.0	0.2	0.2
2	start	2000	9.8	196.0	20.0			
	end	2000				784.0	0.4	0.6
	waste	1800				705.6		
3	start	1800	13.6	244.8	25.0			
	end	1800				979.2	0.5	1.1
	waste	1600				870.4		
4	start	1600	15.3	244.8	25.0			
	end	1600				979.2	0.6	1.7
	waste	1400				856.8		
5	start	1400	17.5	244.3	25.0			
	end	1400				977.2	0.7	2.4
	waste	1200				837.6		
6	start	1200	20.4	244.8	25.0			
	end	1200				979.2	0.8	3.3
	waste	1000				816.0		

[a] Start = addition of PCE; end = when the PCE was consumed; waste = removal of 200 mL of culture; [b] Based on feeding schedule used by Eaddy (8); [c] (µmol/100 mL)*(mL/bottle); [d] Volume of PCE added (µL) = (x µmol PCE/bottle)*(166 µg PCE/1 µmole PCE)*(1 mg/1000 µg)*(1 µL PCE/1.623 mg PCE), where x is the value in the column "µmol/bottle"); [e] (µmol PCE/bottle)*(2 µmol Cl^-/µmol PCE); [f] (µmol Cl^-/bottle)/(mL/bottle); [g] Cumulative Cl^- present = (previous amount) + (amount formed based on footnote *f*).

Table 2.3 Experimental procedure for the treatments receiving 1,2-DCA as TEA.

			1,2-DCA Added			Cl^- Present		
Dose	Operating point[a]	Volume (mL)	µmol/100 mL[b]	µmol/bottle[c]	µL (neat)[d]	µmol/bottle[e]	µmol/mL[f]	Total (µmol/mL)[g]
1	start	2,000	6.3	126.0	10.0	0.0	0.0	0.0
	end	2,000				252.0	0.1	0.1
2	start	2,000	9.5	190.0	15.0			
	end	2,000				380.0	0.2	0.3
3	start	2,000	15.8	316.0	25.0			
	end	2,000				632.0	0.3	0.6
	waste	1,800				568.8		
4	start	1,800	31.6	569.2	45.0			
	end	1,800				1,138.3	0.6	1.3
	waste	1,600				1,011.8		
5	start	1,600	35.6	569.6	45.0			
	end	1,600				1,139.2	0.7	2.0
	waste	1,400				996.8		
6	start	1,400	40.7	569.8	45.0			
	end	1,400				1,139.6	0.8	2.8
	waste	1,200				976.8		
7	start	1,200	47.5	570.0	45.0			
	end	1,200				1,140.0	1.0	3.7
	waste	1,000				977.1		

[a] Start = addition of 1,2-DCA; end = when the 1,2-DCA was consumed; waste = removal of 200 mL of culture; [b] Based on feeding schedule used by Eaddy (8); [c] (µmol/100 mL)*(mL/bottle); [d] Volume of 1,2-DCA added (µL) = (x µmol 1,2-DCA/bottle)*(98.96 µg 1,2-DCA/1 µmole 1,2-DCA)*(1 mg/1000 µg)*(1 µL 1,2-DCA/1.253 mg 1,2-DCA), where x is the value in the column "µmol/bottle"); [e] (µmol 1,2-DCA/bottle)*(2 µmol Cl^-/µmol 1,2-DCA); [f] (µmol Cl^-/bottle)/(mL/bottle); [g] Cumulative Cl^- present = (previous amount) + (amount formed based on footnote *f*).

Table 2.4 Experimental procedure for the treatments receiving EDB as TEA.

			EDB Added			Br^- Present		
Dose	Operating point[a]	Volume (mL)	µmol/100 mL[b]	µmol/bottle[c]	µL (neat)[d]	µmol/bottle[e]	µmol/ mL[f]	Total (µmol/mL)[g]
1	start	2,000	5.8	116.0	10.0	0.0	0.0	0.0
	end	2,000				232.0	0.1	0.1
2	start	2,000	11.6	231.0	20.0			
	end	2,000				462.0	0.2	0.3
3	start	2,000	17.3	346.0	30.0			
	end	2,000				692.0	0.3	0.7
	waste	1,800				622.8		
4	start	1,800	28.9	520.2	45.0			
	end	1,800				1,040.4	0.6	1.3
	waste	1,600				924.8		
5	start	1,600	32.5	520.0	45.0			
	end	1,600				1,040.0	0.7	1.9
	waste	1,400				910.0		
6	start	1,400	37.1	519.4	45.0			
	end	1,400				1,038.8	0.7	2.7
	waste	1,200				890.4		
7	start	1,200	43.3	519.5	45.0			
	end	1,200				1,039.0	0.9	3.5
	waste	1,000				890.5		

[a] Start = addition of EDB; end = when the EDB was consumed; waste = removal of 200 mL of culture; [b] Based on feeding schedule used by Eaddy (8); [c] (µmol/100 mL)*(mL/bottle); [d] Volume of EDB added (µL) = (x µmol EDB/bottle)*(187.86 µg EDB/1 µmole EDB)*(1 mg/1000 µg)*(1 µL EDB/2.17 mg EDB), where x is the value in the column "µmol/bottle"); [e] (µmol EDB/bottle)*(2 µmol Br^-/µmol EDB); [f] (µmol Br^-/bottle)/(mL/bottle); [g] Cumulative Br^- present = (previous amount) + (amount formed based on footnote *f*).

Table 2.5 qPCR primers and probes used in this study.

Name	Primer sequence (5'-3')	Specificity	Reference
DHC 1200F	ctggagctaatccccaaagct	*Dehalococcoides* spp.	(17)
DHC 1271R	caacttcatgcaggcggg	*Dehalococcoides* spp.	(17)
DHC 1240Probe	FAM-tcctcagttcggattgcaggctgaa-TAMRA	*Dehalococcoides* spp.	(17)
DHB 406F	gttagggaagaacggcatctgt	*Dehalobacter* spp.	(33)
DHB 645R	cctctcctgtcctcaagccata	*Dehalobacter* spp.	(33)
DSB 406F	gtacgacgaaggccttcgggt	*Desulfitobacterium* spp.	(33)
DSB 619R	cccagggttgagccctaggt	*Desulfitobacterium* spp.	(33)

Table 3.1 Recovery percentages for daughter products.

		Daughter product formed (mol %)							
Treatment	Bottle	TCE	cDCE	BA	CA	VB	VC	Ethene	Loss
SRS Mother		**0.0%**	**0.2%**	**-**	-	**-**	**0.2%**	**86.8%**	**12.8%**
DCA Mother		-	-	**-**	**0.0%**	**-**	**0.0%**	**92.3%**	**7.7%**
EDB Mother		-	-	**0.0%**	-	**0.0%**	-	**89.4%**	**10.6%**
#1 (PCE/SRS Mother)	1	0.0%	0.1%	-	-	-	0.0%	98.0%	1.9%
	2	0.0%	0.0%	-	-	-	0.0%	96.7%	3.3%
	average	**0.0%**	**0.1%**				**0.0%**	**97.3%**	**2.6%**
#2 (1,2-DCA/DCA Mother)	1	-	-	-	0.0%	-	0.0%	92.9%	7.1%
	2	-	-	-	0.0%	-	0.0%	96.4%	3.6%
	average				**0.0%**		**0.0%**	**94.7%**	**5.3%**
#5 (1,2-DCA/EDB Mother)	1	-	-	-	0.0%	-	0.0%	97.8%	2.2%
	2	-	-	-	0.0%	-	0.0%	98.3%	1.7%
	average				**0.0%**		**0.0%**	**98.1%**	**1.9%**
#4 (EDB/DCA Mother)	1	-	-	0.0%	-	0.0%	-	94.9%	5.1%
	2	-	-	0.0%	-	0.0%	-	93.5%	6.5%
	average			**0.0%**		**0.0%**		**94.2%**	**5.8%**
#3 (EDB/EDB Mother)	1	-	-	0.0%	-	0.0%	-	90.4%	9.6%
	2	-	-	0.0%	-	0.0%	-	90.6%	9.4%
	average			**0.0%**		**0.0%**		**90.5%**	**9.5%**

Table 3.2 Summary of lactate added, methanogenic and dehalogenation electron equivalents.

		Lactate Added			Methane			Dehalogenation	
Treatment	Bottle	mL	mmol/bottle	meq/bottle	mmol/bottle	meq/bottle	%	meq/bottle	%
SRS to PCE	#1	38	142.607	1711.281	3.497	27.977	1.63%	9.750	0.6%
	#2	38	142.607	1711.281	2.706	21.649	1.27%	9.651	0.6%
	average		**142.607**	**1711.281**	**3.102**	**24.813**	**1.45%**	**9.701**	**0.6%**
DCA to 1,2-DCA	#1	31	117.838	1414.058	0.762	6.097	0.43%	5.776	0.4%
	#2	29	110.333	1323.991	1.413	11.300	0.85%	5.910	0.4%
	average		**114.085**	**1369.025**	**1.087**	**8.699**	**0.64%**	**5.843**	**0.4%**
EDB to EDB	#1	31	116.337	1396.045	0.018	0.143	0.01%	5.901	0.4%
	#2	31	116.337	1396.045	0.028	0.222	0.02%	5.992	0.4%
	average		**116.337**	**1396.045**	**0.023**	**0.182**	**0.01%**	**5.947**	**0.4%**
DCA to EDB	#1	27	99.825	1197.897	0.029	0.235	0.02%	5.970	0.5%
	#2	27	102.827	1233.924	0.309	2.469	0.20%	6.369	0.5%
	average		**101.326**	**1215.910**	**0.169**	**1.352**	**0.11%**	**6.170**	**0.5%**
EDB to 1,2-DCA	#1	31	114.836	1378.031	3.272	26.177	1.90%	6.096	0.4%
	#2	31	114.836	1378.031	4.436	35.488	2.58%	6.259	0.5%
	average		**114.836**	**1378.031**	**3.854**	**30.832**	**2.24%**	**6.177**	**0.4%**
DCA mother		9	33.775	**405.303**	**0.536**	**4.287**	**1.06%**	**4.353**	**1.1%**
EDB mother		7	26.270	**315.236**	**0.161**	**1.289**	**0.41%**	**3.696**	**1.2%**
SRS mother		20	75.056	**900.674**	**0.188**	**1.508**	**0.17%**	**27.640**	**3.1%**

Table 3.3 *Dehalobacter* concentrations determined by qPCR.

Treatment	Bottle No.	*Dehalobacter* (gene copies/mL)	
		Average	Standard deviation
DCA Mother	-	BD[a]	-
EDB Mother	-	5.10E+03	5.68E+02
SRS Mother	-	BD	-
#1 (PCE/SRS Mother)	1	4.15E+03	1.41E+03
	2	6.33E+03	1.14E+03
#2 (1,2-DCA/DCA Mother)	1	BD	-
	2	BD	-
#3 (EDB/EDB Mother)	1	3.07E+04	2.96E+03
	2	2.29E+04	1.87E+03
#5 (1,2-DCA/EDB Mother)	1	6.53E+03	4.37E+02
	2	7.55E+03	1.66E+03

[a] BD = below detection.

Table 4.1 Growth yields for *Dehalococcoides*.

Organism/culture	Electron acceptor	Yield (copy number/μmol Cl^-)	Primers	Detection Method	Reference
Isolate BAV1	VC	$6.3\pm0.3\times10^7$	DHC1200F,1271R	Taqman	(18)
KB-1/VC	VC	$(5.6\pm1.4)\times10^8$	DHC 1F and 259R	SYBR	(7)
Mixed culture	VC	$(8.68\pm2.62)\times10^7$	DHC 1F and 264R	SYBR	(15)
ANAS	VC	$(1.3\pm0.3)\times10^7$	[a]		(22)
Isolate GT	VC	$(2.5\pm0.13)\times10^8$	DHC1200F,1271R	Taqman	(31)
KB-1/TCE	VC	$(2.9\pm0.6)\times10^8$	DHC 1F and 259R	SYBR	(7)
Isolate FL2	cDCE	$(8.4\pm0.8)\times10^7$	DHC1200F,1271R	Taqman	(19)
ANAS	cDCE	$(1.1\pm0.1)\times10^7$	[a]		(22)
KB-1/TCE	cDCE	$(1.75\pm1.3)\times10^8$	DHC 1F and 259R	SYBR	(7)
KB-1/VC	TCE	$(3.6\pm1.3)\times10^8$	DHC 1F and 259R	SYBR	(7)
Isolate FL2	TCE	$(7.8\pm0.9)\times10^7$	DHC1200F,1271R	Taqman	(19)
ANAS	TCE	$(1.4\pm0.4)\times10^7$	[a]		(22)
Isolate GT	TCE	$(2.3\pm0.72)\times10^8$	DHC1200F,1271R	Taqman	(31)
SRS enrichment	PCE	$(2.11\pm0.082)\times10^8$	DHC1200F,1271R	Taqman	This
Modeling	PCE	6.9×10^8 [b]			(2)
KB-1/TCE	1,2-	$(1.6\pm1.5)\times10^8$	DHC 1F and 259R	SYBR	(7)
DCA mother	1,2-	$(4.59\pm0.036)\times10^7$	DHC1200F,1271R	Taqman	This
EDB mother	1,2-	$(2.57\pm0.013)\times10^7$	DHC1200F,1271R	Taqman	This
EDB mother	EDB	$(7.13\pm0.63)\times10^6$	DHC1200F,1271R	Taqman	This

[b] Estimated based on assumptions used by Duhamel and Edwards (7).
[a] Forward primer, 5'-GGTAATACGTAGGGAAGCAAGCG; probe, 5'-ACATCCAACTTGAAAGACCACCTACGCTCACT; and reverse, 5'-CCGGTTAAGCCGGGAAATT.

FIGURES

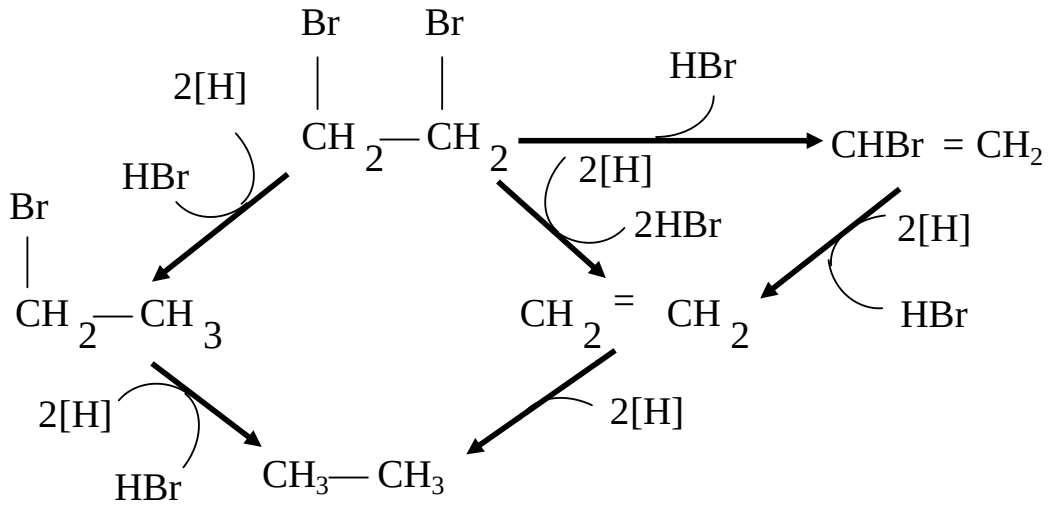

Figure 1.1 Pathways for anaerobic biodegradation of EDB. [H] = $H^+ + e^-$. All steps are the same for 1,2-DCA, when switching Br to Cl.

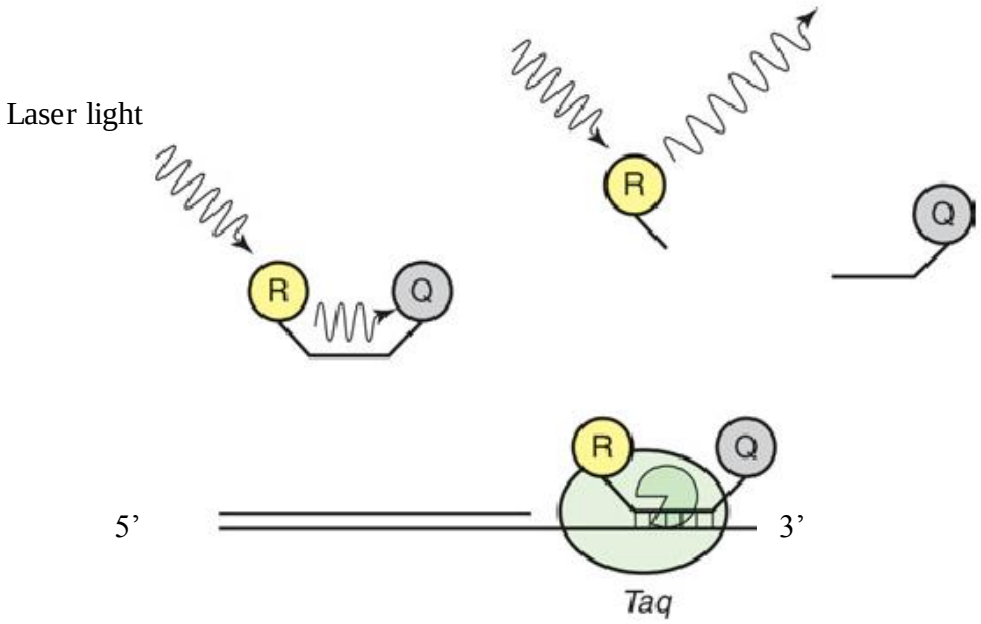

Figure 2.1 Schematic representation of how the TaqMan® probe based qPCR works. *Taq* is the DNA polymerase; R is the reporter fluorophore; and Q is the quencher fluorophore.

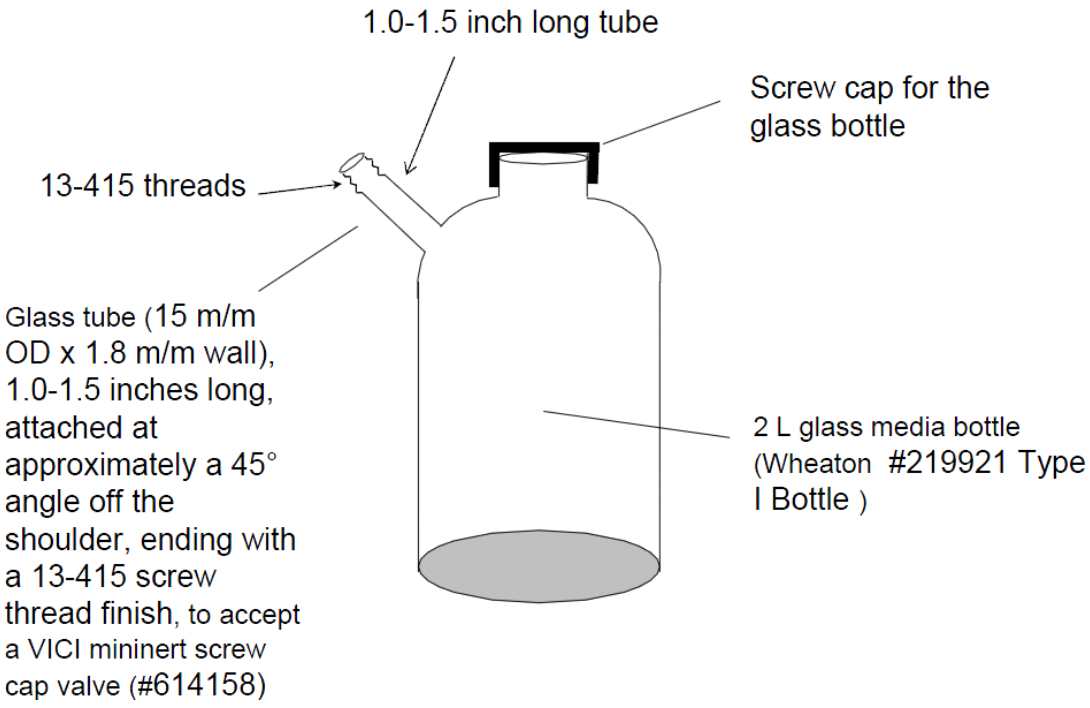

Figure 2.2 2 L modified glass media bottle with Mininert valve (5).

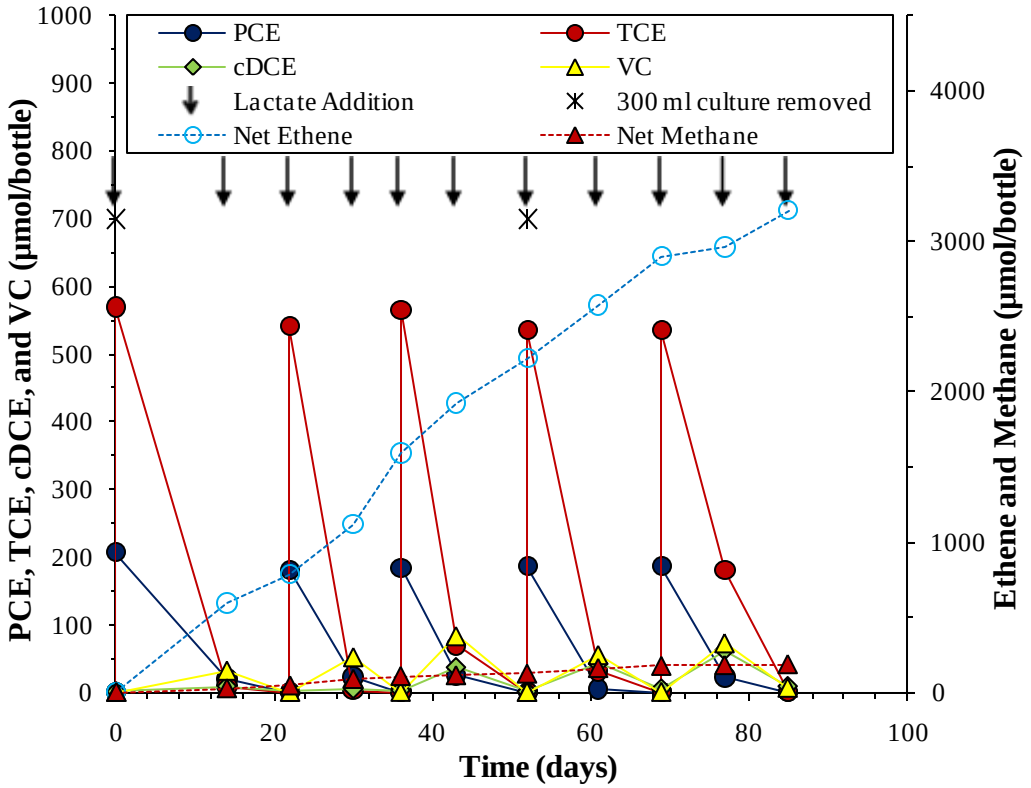

Figure 3.1 Results for the SRS Mother Culture (MicroCED), for the time interval relevant to this project.

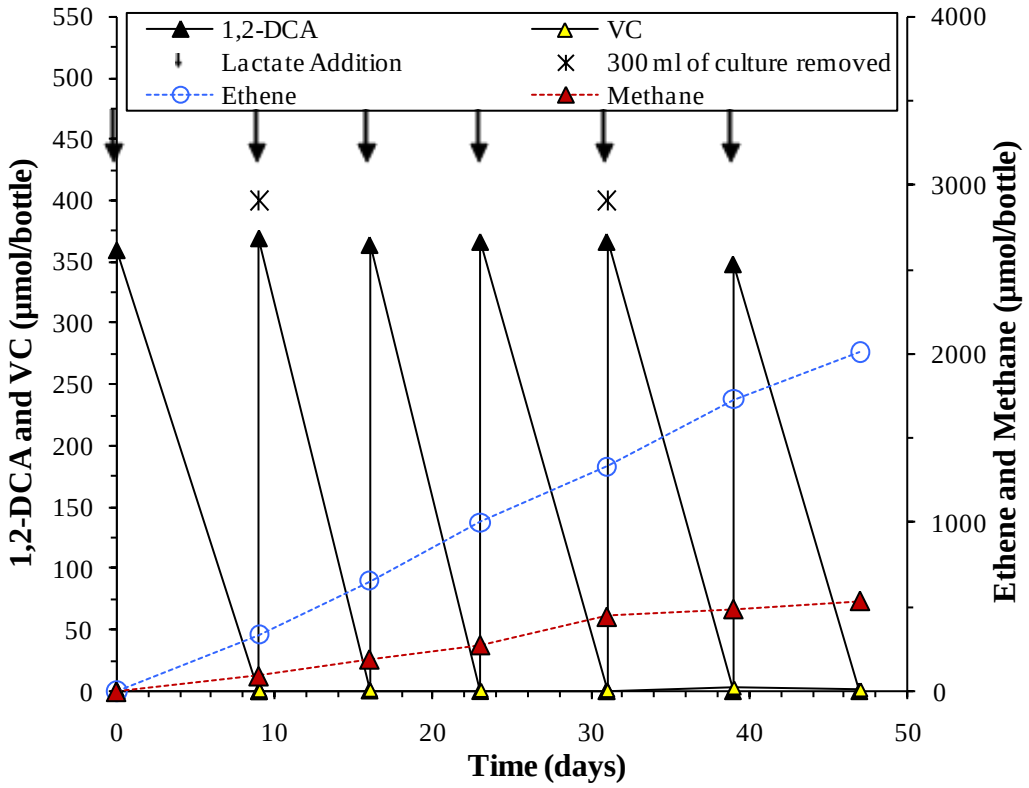

Figure 3.2 Results for the 1,2-DCA Mother Culture, for the time interval relevant to this project.

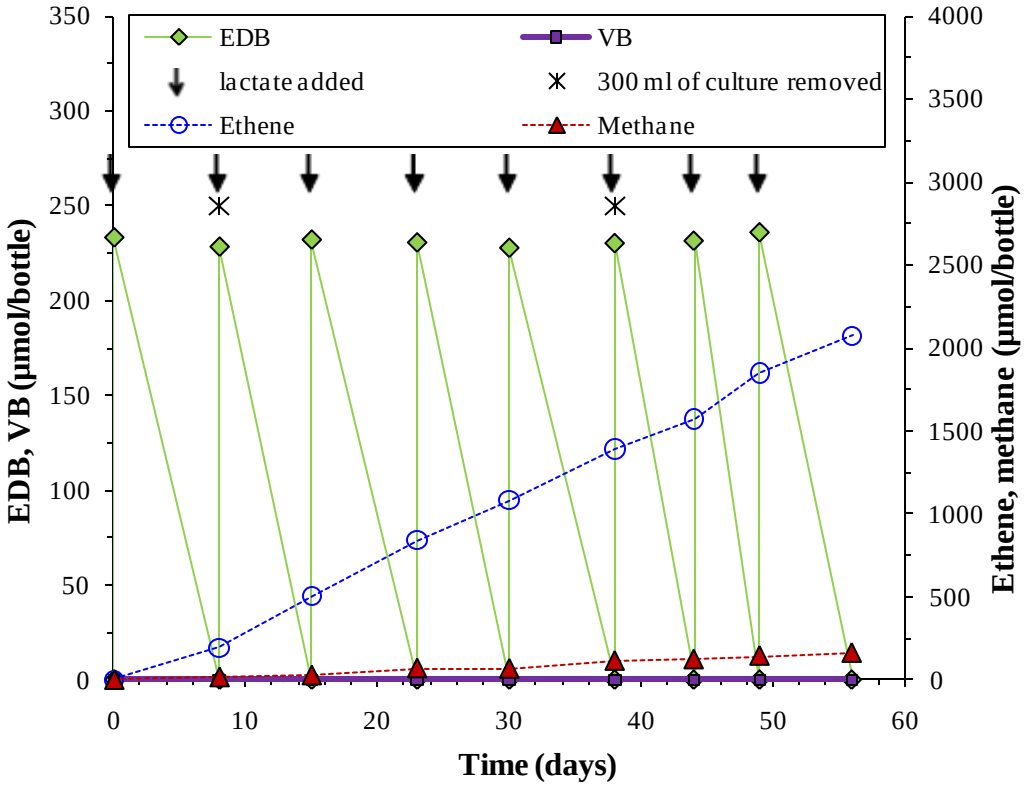

Figure 3.3 Results for the EDB Mother Culture, for the time interval relevant to this project.

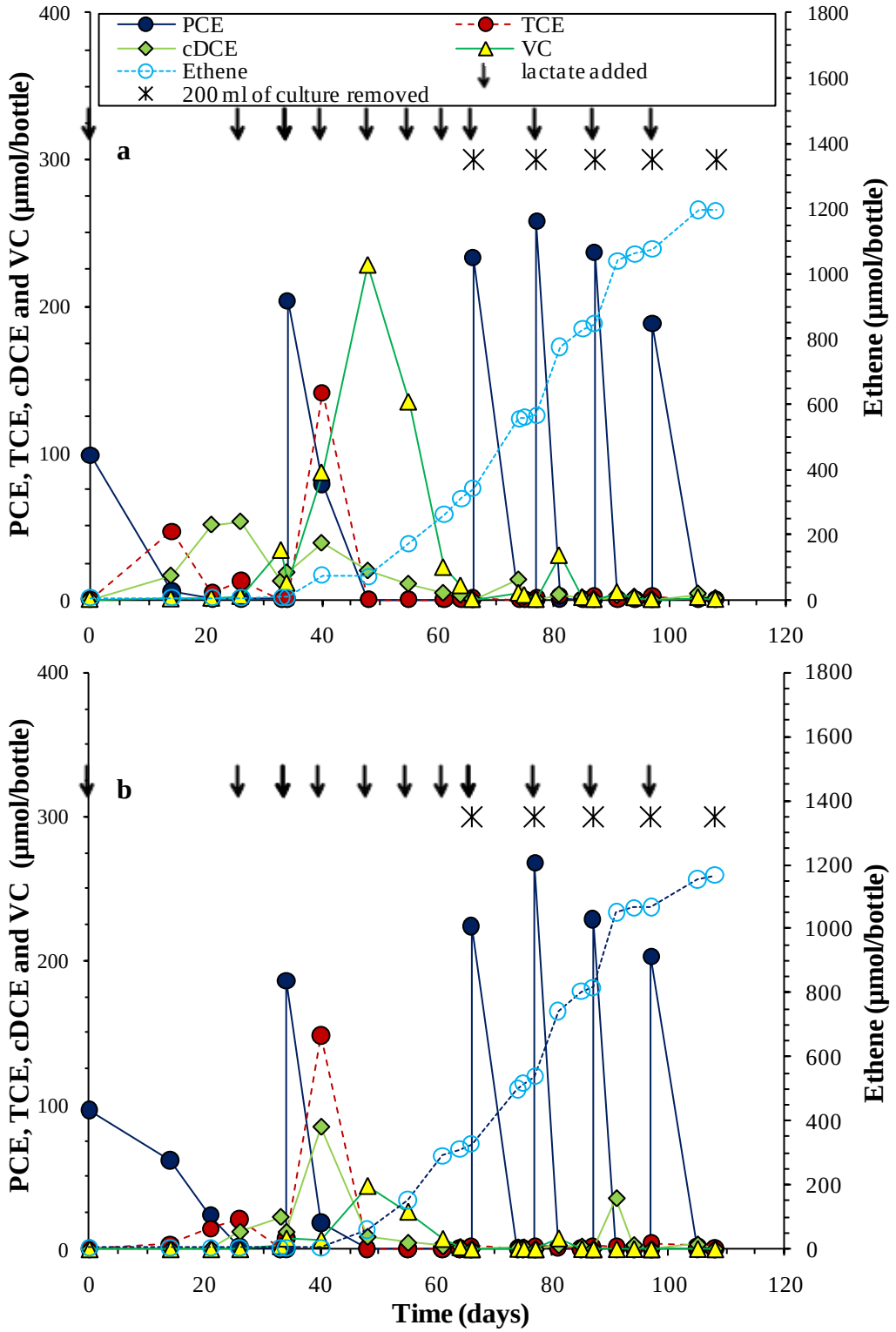

Figure 3.4 Results for treatment #1, SRS Mother Culture fed with PCE; a) bottle #1; b) bottle #2.

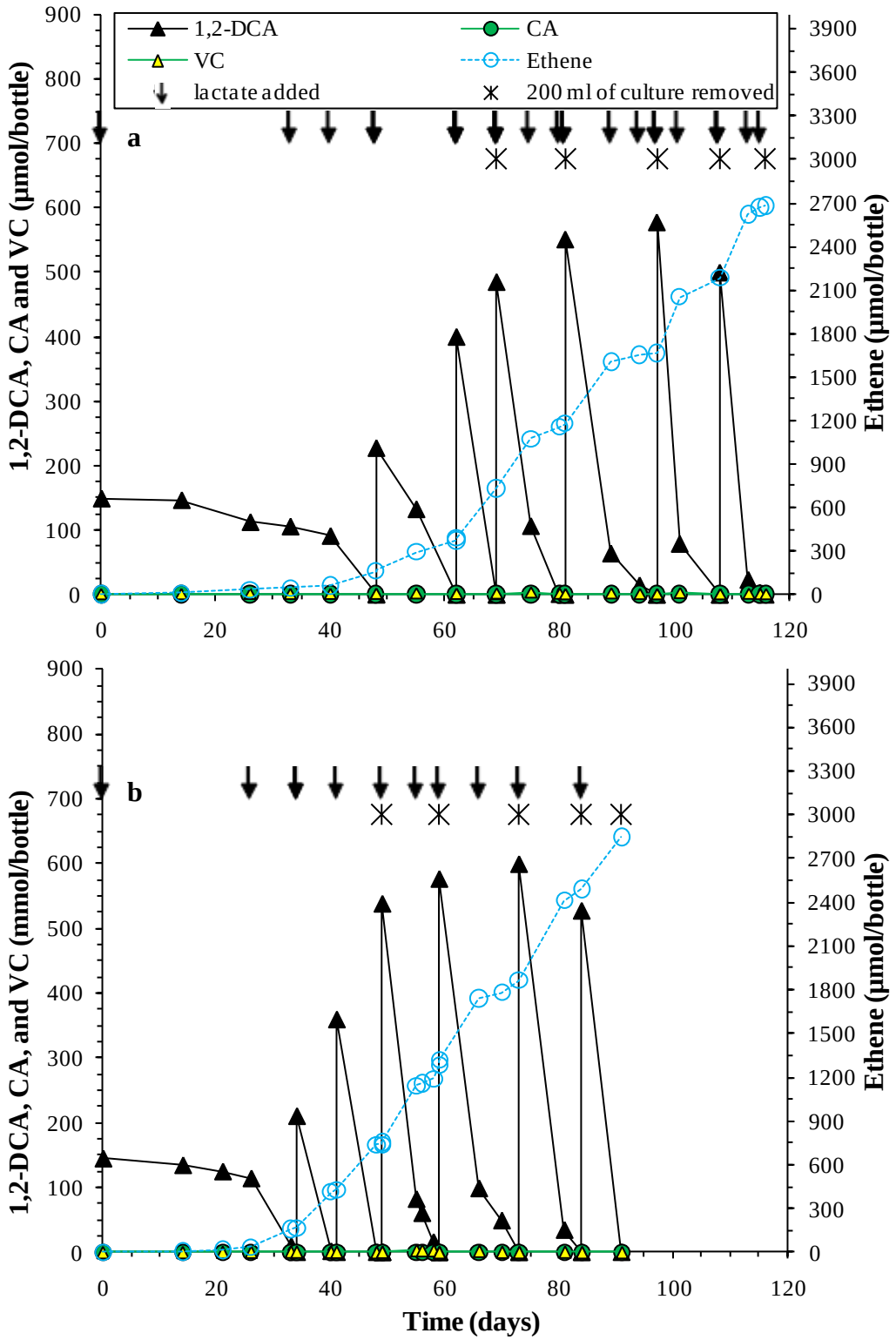

Figure 3.5 Results for treatment #2, DCA Mother Culture fed with 1,2-DCA; a) bottle #1; b) bottle #2.

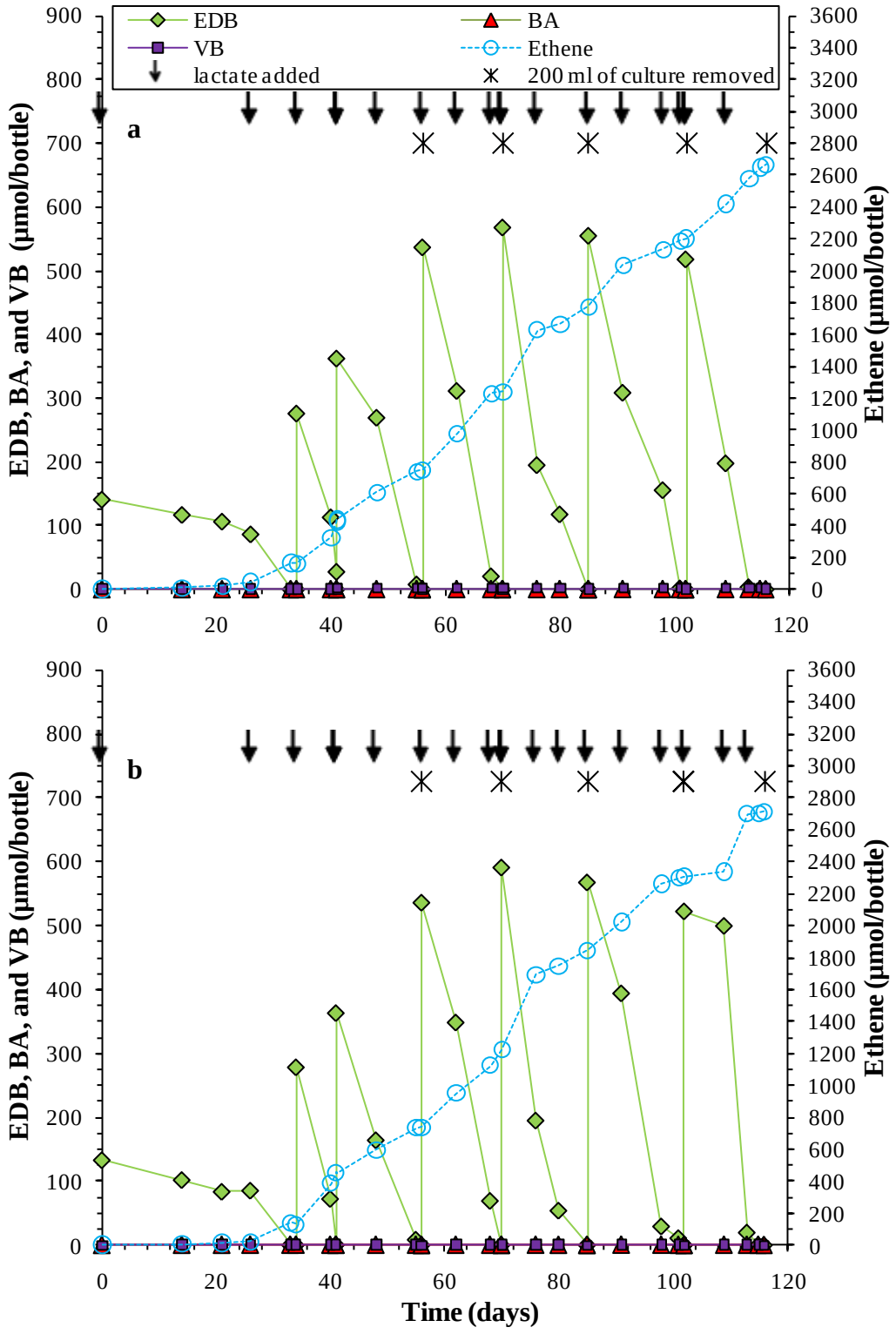

Figure 3.6 Results for treatment #3, EDB Mother Culture fed with EDB, a) bottle #1; b) bottle #2.

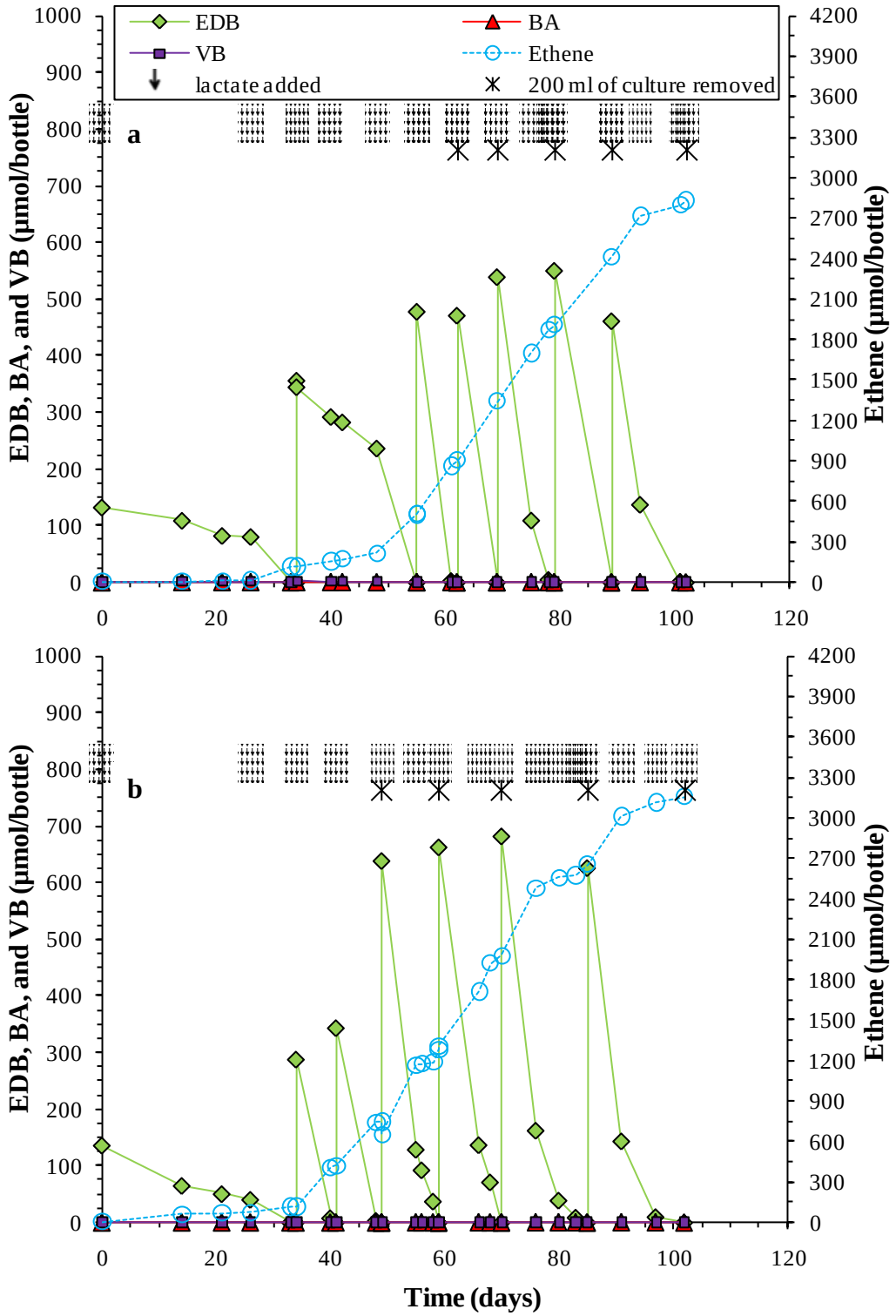

Figure 3.7 Results for treatment #4, DCA Mother Culture fed with EDB, a) bottle #1; b) bottle #2.

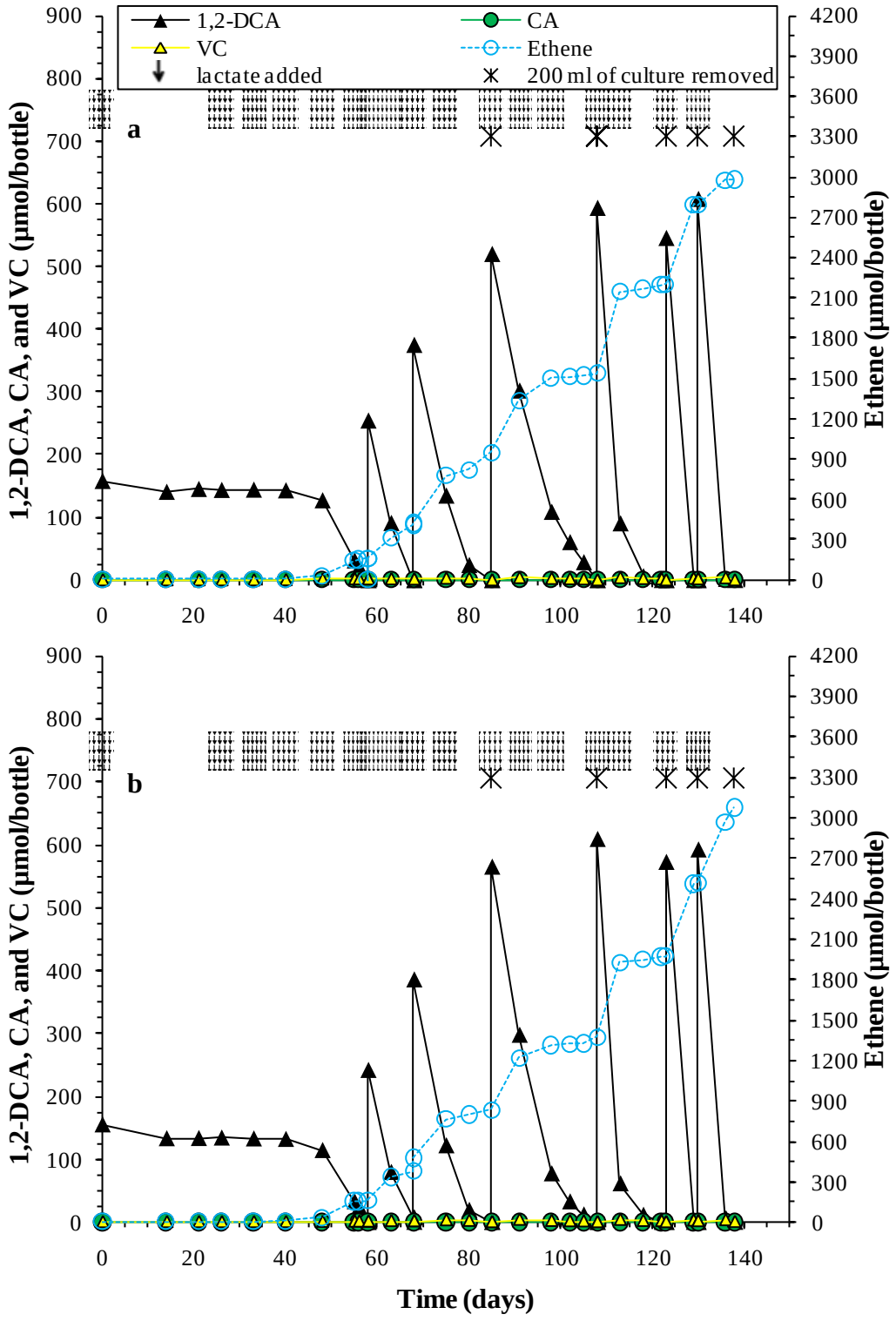

Figure 3.8 Results for treatment #5, EDB Mother Culture fed with 1,2-DCA, a) bottle #1; b) bottle #2.

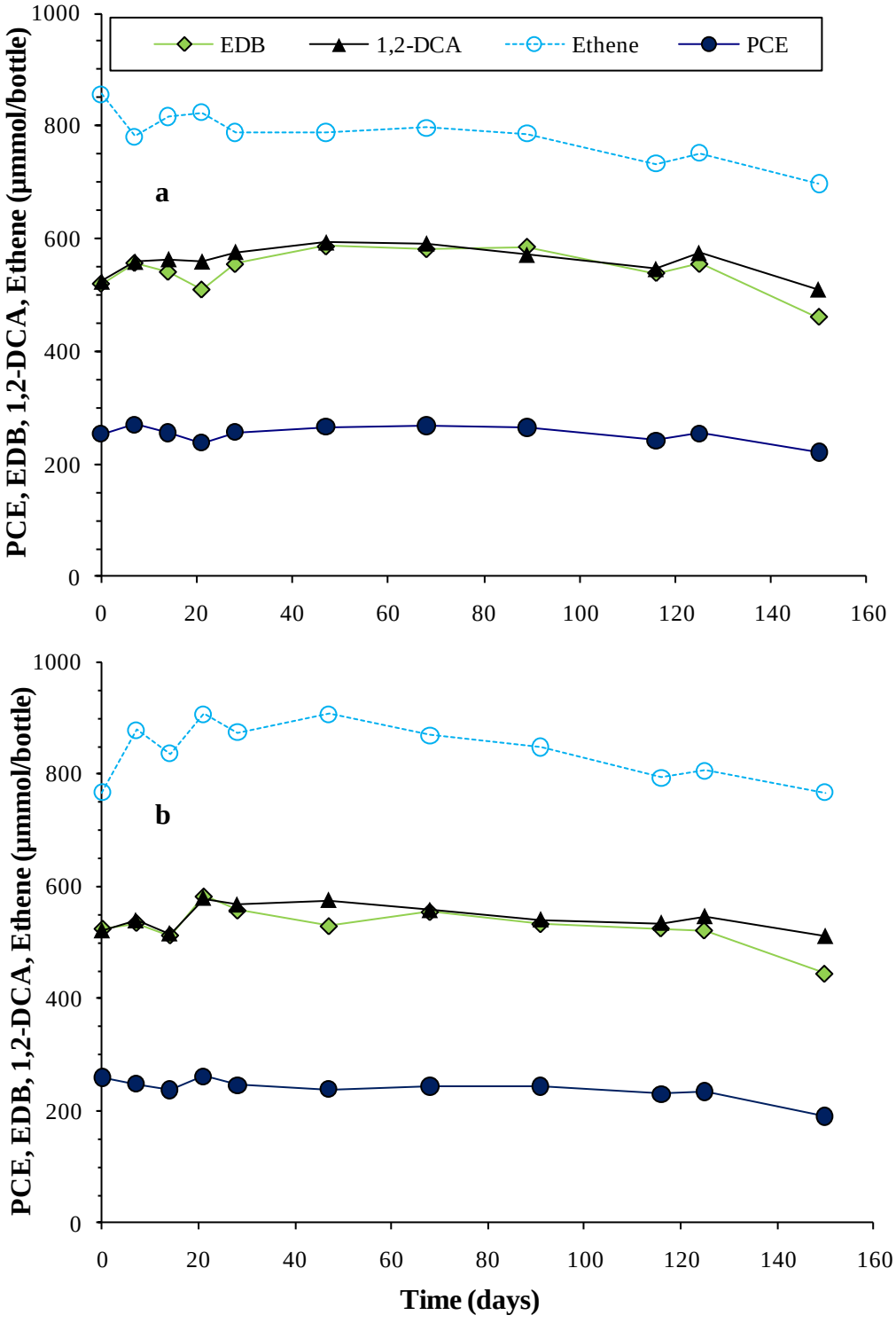

Figure 3.9 Results for WCs, **a)** bottle #1; b) bottle #2.

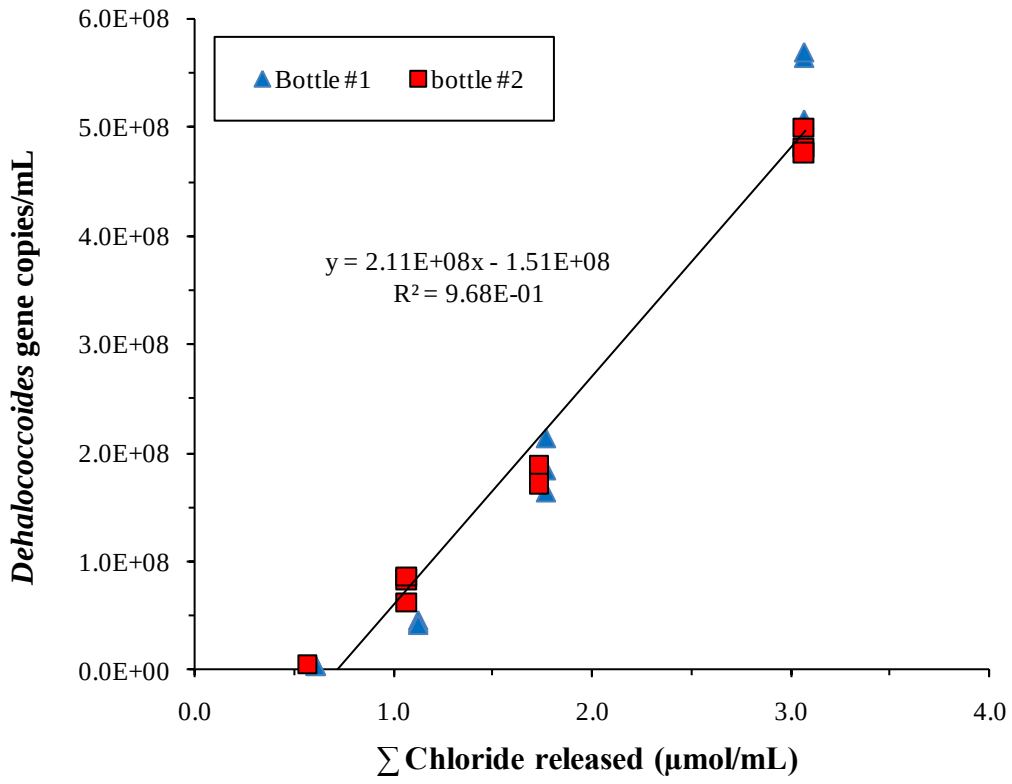

Figure 3.10 qPCR and chloride release results for the experimental bottle inoculated with the SRS Mother Culture and fed PCE (treatment #1 in Table 2.1). Data point for fourth stage of this experiment were removed, since the copy numbers were consistently lower than the third and second stage.

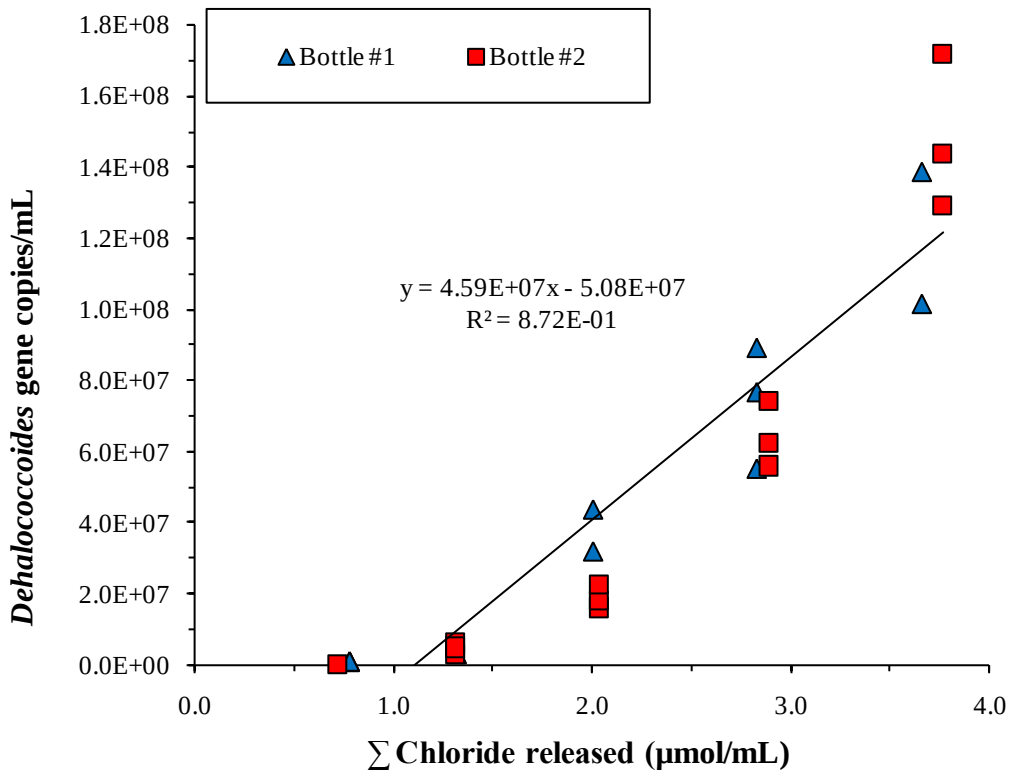

Figure 3.11 qPCR and chloride release results for the experimental bottle inoculated with the DCA Mother Culture and fed 1,2-DCA (treatment #2 in Table 2.1).

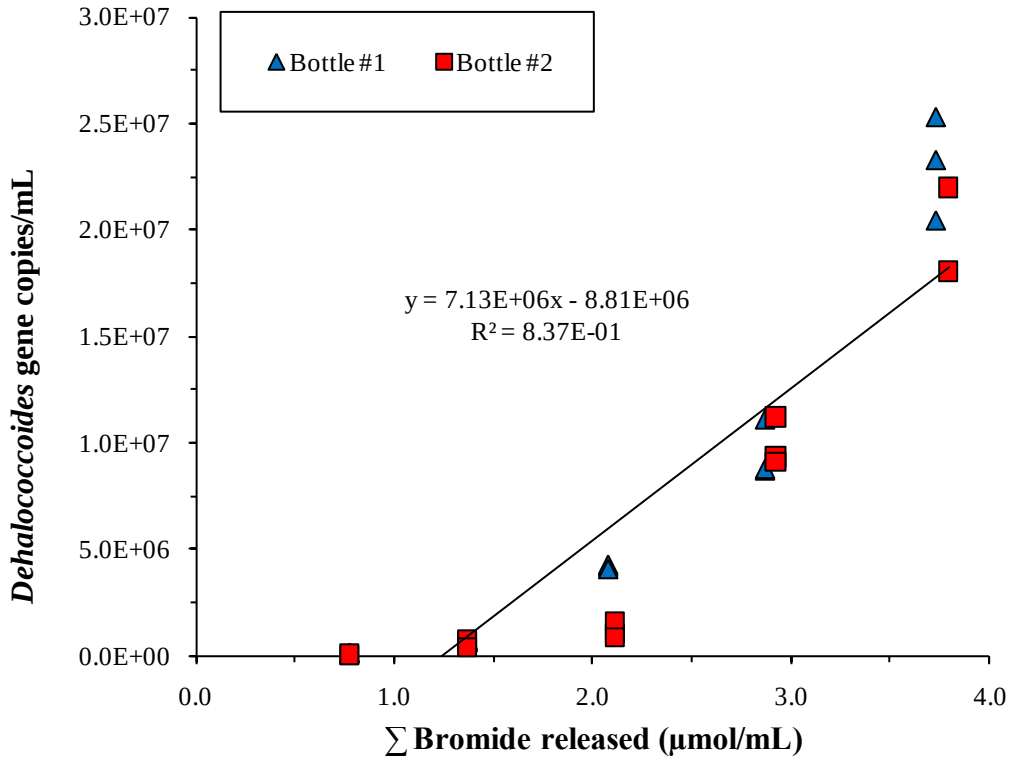

Figure 3.12 qPCR and bromide release results for the experimental bottle inoculated with the EDB Mother Culture and fed EDB (treatment #3 in Table 2.1).

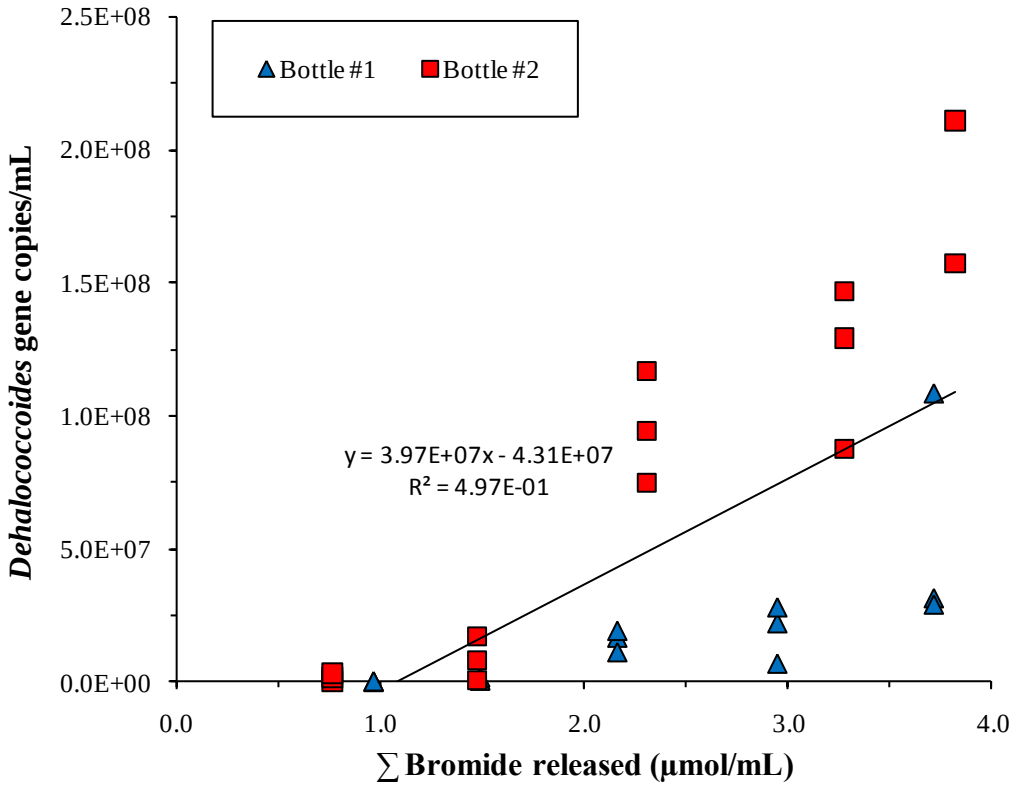

Figure 3.13 qPCR and bromide release results for the experimental bottle inoculated with the DCA Mother Culture and fed EDB (treatment #4 in Table 2.1).

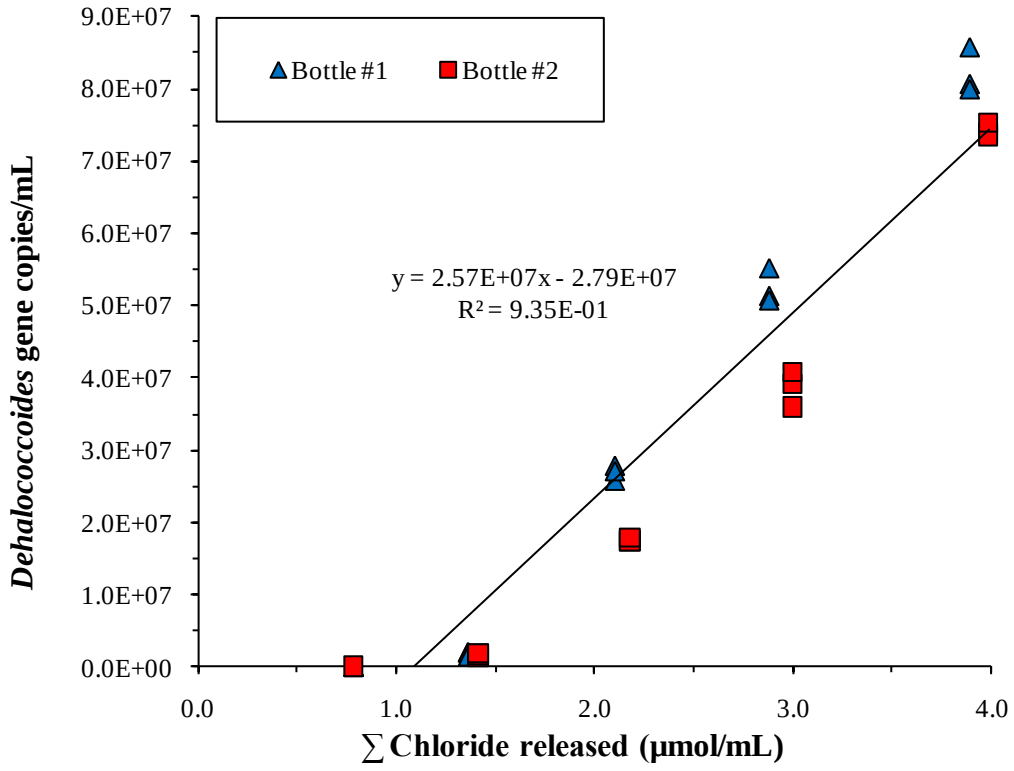

Figure 3.14 qPCR and chloride release results for the experimental bottle inoculated with the EDB Mother Culture and fed 1,2-DCA (treatment #5 in Table 2.1).

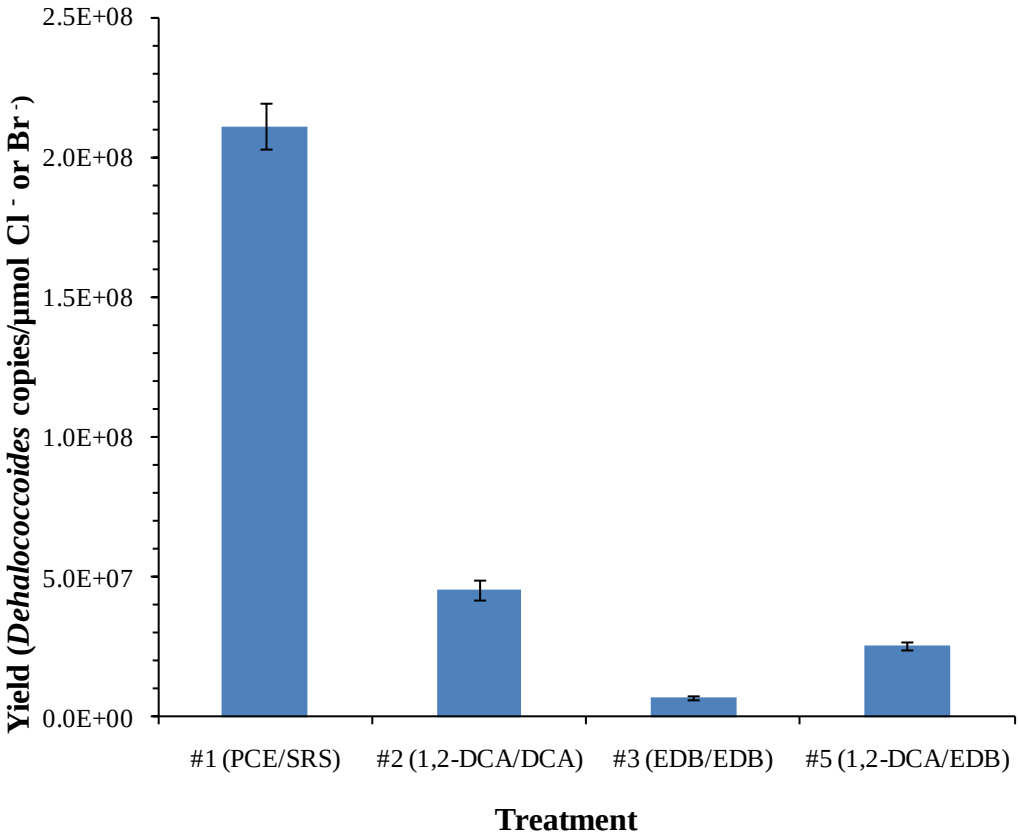

Figure 3.15 Yield values for *Dehalococcoides*; each bar represents the average for pooled data shown in Figures 3.10-3.14; error bars represent the standard error for the regression line used to determine Y.

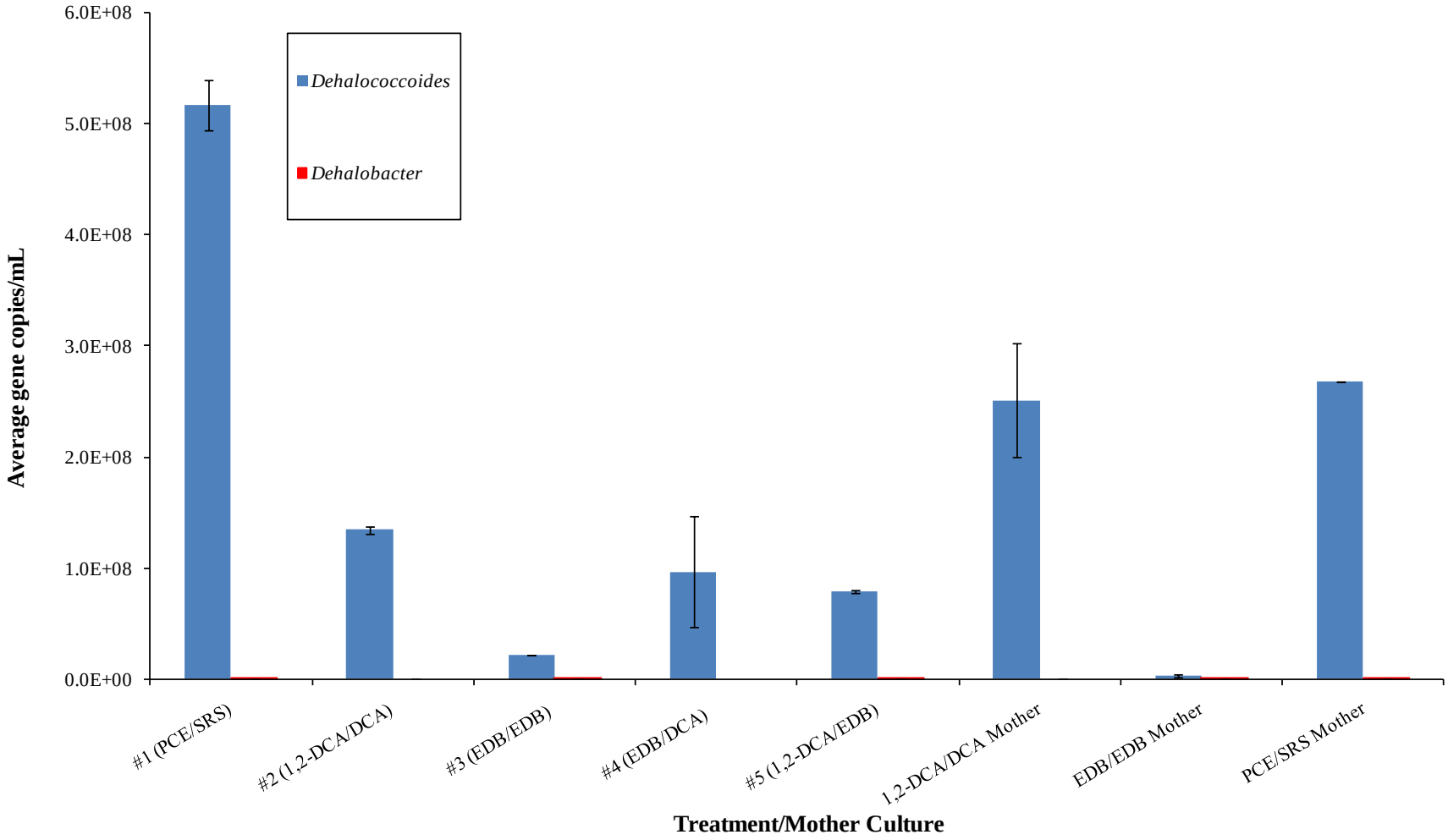

Figure 3.16 Comparison of *Dehalococcoides* and *Dehalobacter* concentrations in the experimental bottles (final sample) and Mother Cultures. Each bar is the average of triplicate qPCR analyses; averages for the experimental treatments include duplicate bottles. Error bars represent one standard deviation.

APPENDICES

Appendix A: Preparation of Enrichment Culture Media

Solutions needed for media:

- Phosphate buffer

  In a 100 mL volumetric flask add 5.25 g K_2HPO_4. Then fill to 100 mL with DDI water.

- Salt solution

  In a 100 mL volumetric flask add 5.35 g NH_4Cl, 0.46976 g $CaCl_2$:$2H_2O$, and 0.17787 g $FeCl_2$:H_2O. Then fill to 100 mL with DDI water.

- Trace metals solution

  In a 100 mL volumetric flask add 0.03 g H_3BO_3, 0.0211 g $ZnSO_4$:$7H_2O$, 0.075 g $NiCl_2$:6H2O, 0.1 g $MnCl_2$:$4H_2O$, 0.01 g $CuCl_2$:$2H_2O$, 0.15 g $CoCl_2$:$6H_2O$, 0.002 g Na_2SeO_3, 0.01 g $Al_2(SO_4)_3$:$16H_2O$, and 1 mL concentrated HCl. Then fill to 100 mL with DDI water.

- Magnesium sulfate solution

  In a 100 mL volumetric flask add 6.25 g $MgSO_4$:$7H_2O$. Then fill to 100 mL with DDI water.

- Bicarbonate solution

  In a 500 mL volumetric flask add 8.0 g $NaHCO_3$. Then fill to 500 mL with DDI water.

- Redox indicator solution

  In a 10 mL volumetric flask add 0.01 g resazurin. Then fill to 10 mL with DDI water.

- Ferrous sulfide solution

  To be added directly to autoclaved media after adding filter-sterilized solutions and placing in glove box. For 1 L, weigh out 0.24g of Na_2S:$9H_2O$ and 0.1448g $FeCl_2$:H_2O based on stock concentrations of 24 g/L and 14.48 g/L, into separate vials. Add the Na_2S:$9H_2O$ and allow media to clear. Add the $FeCl_2$:H_2O and rinse both vials out with the 10 mL of autoclaved DDI water needed to balance the solution and add to the media.

-Yeast extract solution

  In a 100 mL volumetric flask add 0.5 g yeast extract. Then fill to 100 mL with DDI water.

Media Preparation

In a 1 L bottle add 10 mL phosphate solution, 10 mL salt solution, 2 mL trace metals solution, 2 mL magnesium sulfate solution, 1 mL redox solution, and 905 mL DDI water. Autoclave this solution, and then add 50 mL filter sterilized bicarbonate solution and 10 mL filter sterilized yeast extract. In the glove box, add the 10 mL ferrous sulfide solution.

Appendix B: GC Standards and Response Factors

Table B.1 Response factors used for the SRS Mother Culture.[a]

Compound	GC RT[b] (min)	RF (µmol/bottle/PAU[c])	R^2
Methane	0.6	1.8803E-06	9.923E-01
Ethene	0.8	1.1299E-06	9.944E-01
VC	3.2	2.7995E-06	9.881E-01
cDCE	7.4	1.3410E-05	9.957E-01
TCE	10.8	6.4240E-06	9.997E-01
PCE	16.5	3.9840E-06	9.998E-01

[a] Calculated using the response factors determine by Wood (37) for 160 mL serum bottles and multiplied by the ratio of the volume in the Mother Culture bottles (i.e., 2650 mL/160 mL);

[b] RT = retention time;

[c] RF = response factor; PAU = Peak Area Unit.

Table B.2 Response factors for the DCA Mother Culture estimated from Eaddy (8).

Compound	GC RT[a] (min)	RF (µmol/bottle/PAU[b])	R^2
Methane	0.5	1.893E-06	9.997E-01
Ethene	0.81	1.168E-06	9.999E-01
VC	0.98	9.940E-07	9.999E-01
CA	3.2	2.520E-06	9.999E-01
1,2-DCA	3.9	9.940E-07	9.992E-01

[a] RT = retention time;

[b] RF = response factor; PAU = Peak Area Unit.

Table B.3 Response factors for the EDB Mother Culture estimated from Eaddy (8).

Compound	GC RT[a] (min)	RF (µmol/bottle/PAU[b])	R^2
Methane	0.6	1.89E-06	9.999E-01
Ethene	0.86	1.17E-06	9.998E-01
VB	4.9	4.08E-06	9.995E-01
BA	5.6	6.95E-06	9.993E-01
EDB	12.0	8.61E-05	9.999E-01

[a] RT = retention time;

[b] RF = response factor; PAU = Peak Area Unit.

Table B.4 Response Factors for microcosms with 2 L liquid and 0.3 L headspace at 23°C.

Compound	GC RT[a] (min)	RF (µmol/bottle/PAU[b])	R^2
Methane	0.6	2.031E-05[c]	
Ethene	0.8	1.221E-05	9.879E-01
VC	3.2	3.025E-05[d]	
CA	3.9	2.137E-05[e]	
VB	4.9	8.059E-05[f]	
BA	5.6	1.372E-04[g]	
cDCE	7.4	2.173E-04[h]	
1,2-DCA	8.1	8.808E-04	9.994E-01
TCE	10.8	1.099E-04[i]	
EDB	12.1	1.701E-03	9.927E-01
PCE	16.5	6.819E-05	9.893E-01

[a] RT = retention time;

[b] RF = response factor; PAU = Peak Area Unit;

[c] Methane RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(methane RF for SRS mother bottle);

[d] VC RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(VC RF for SRS mother bottle);

[e] CA RF = (1,2-DCA RF for 2 L bottle)/(1,2-DCA RF for DCA mother bottle)*(CA RF for DCA mother bottle);

[f] VB RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(VB RF for EDB mother bottle);

[g] BA RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(BA RF for EDB mother bottle);

[h] cDCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(cDCE RF for SRS mother bottle);

[i] TCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(TCE RF for SRS mother bottle).

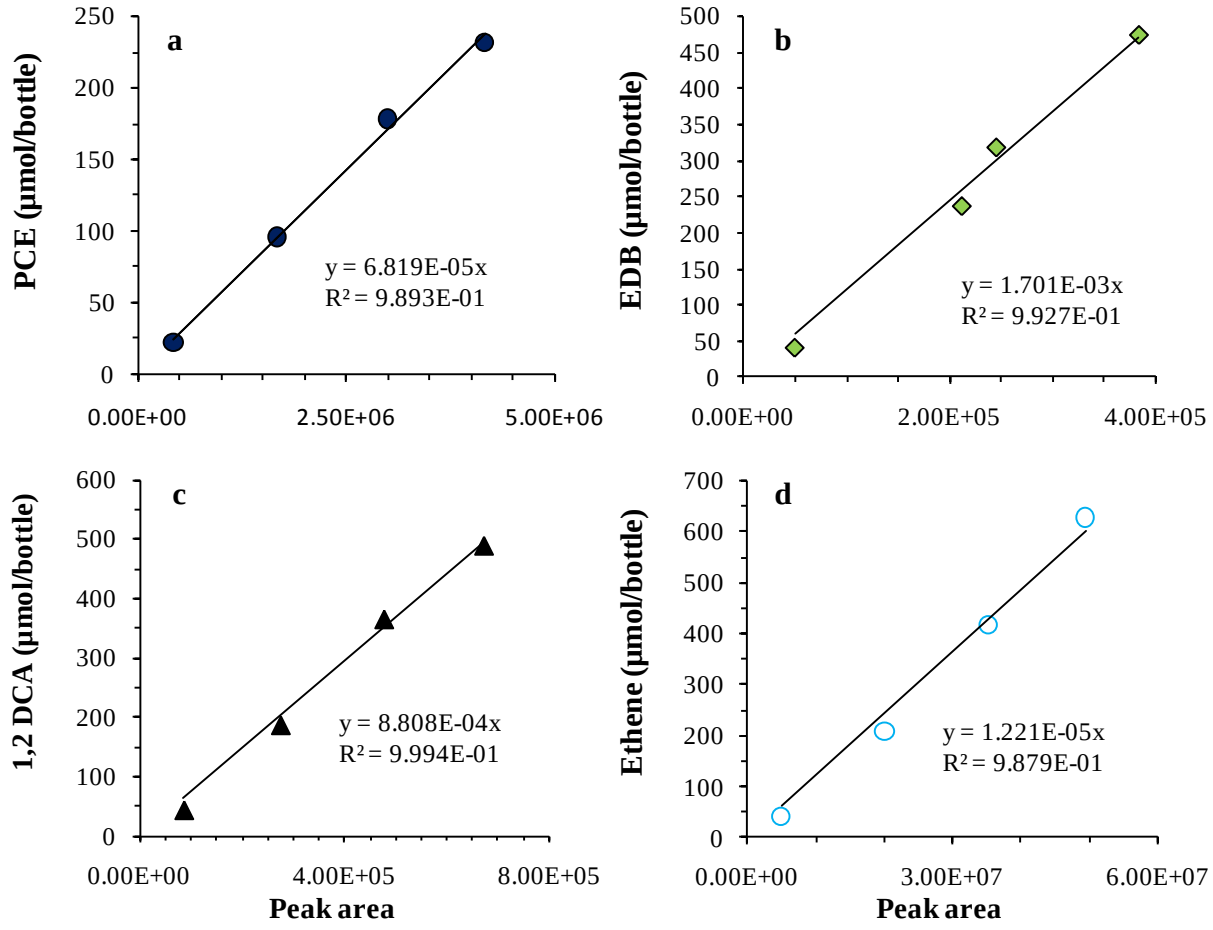

Figure B.1 GC FID response curves for (a) PCE; (b) EDB; (c) 1,2-DCA; and (d) ethene (2 L liquid and 0.3 L headspace at 23°C).

Table B.5 Response factors for microcosms with 2 L liquid and 0.3 L headspace at 23°C.

Compound	GC RT[a] (min)	RF (µmol/bottle/PAU[b])	R^2
Methane	0.6	2.065E-05	
Ethene	0.8	1.251E-05	9.913E-01
VC	3.2	3.075E-05	
CA	3.9	1.862E-05	
VB	4.9	7.084E-05	
BA	5.6	1.206E-04	
cDCE	7.4	2.038E-04	
1,2-DCA	8.1	7.676E-04	9.987E-01
TCE	10.8	1.031E-04	
EDB	12.1	1.535E-03	9.911E-01
PCE	16.5	6.395E-05	9.963E-01

[a] RT = retention time;

[b] RF = response factor; PAU = Peak Area Unit;

[c] Methane RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(methane RF for SRS mother bottle);

[d] VC RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(VC RF for SRS mother bottle);

[e] CA RF = (1,2-DCA RF for 2 L bottle)/(1,2-DCA RF for DCA mother bottle)*(CA RF for DCA mother bottle);

[f] VB RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(VB RF for EDB mother bottle);

[g] BA RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(BA RF for EDB mother bottle);

[h] cDCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(cDCE RF for SRS mother bottle);

[i] TCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(TCE RF for SRS mother bottle).

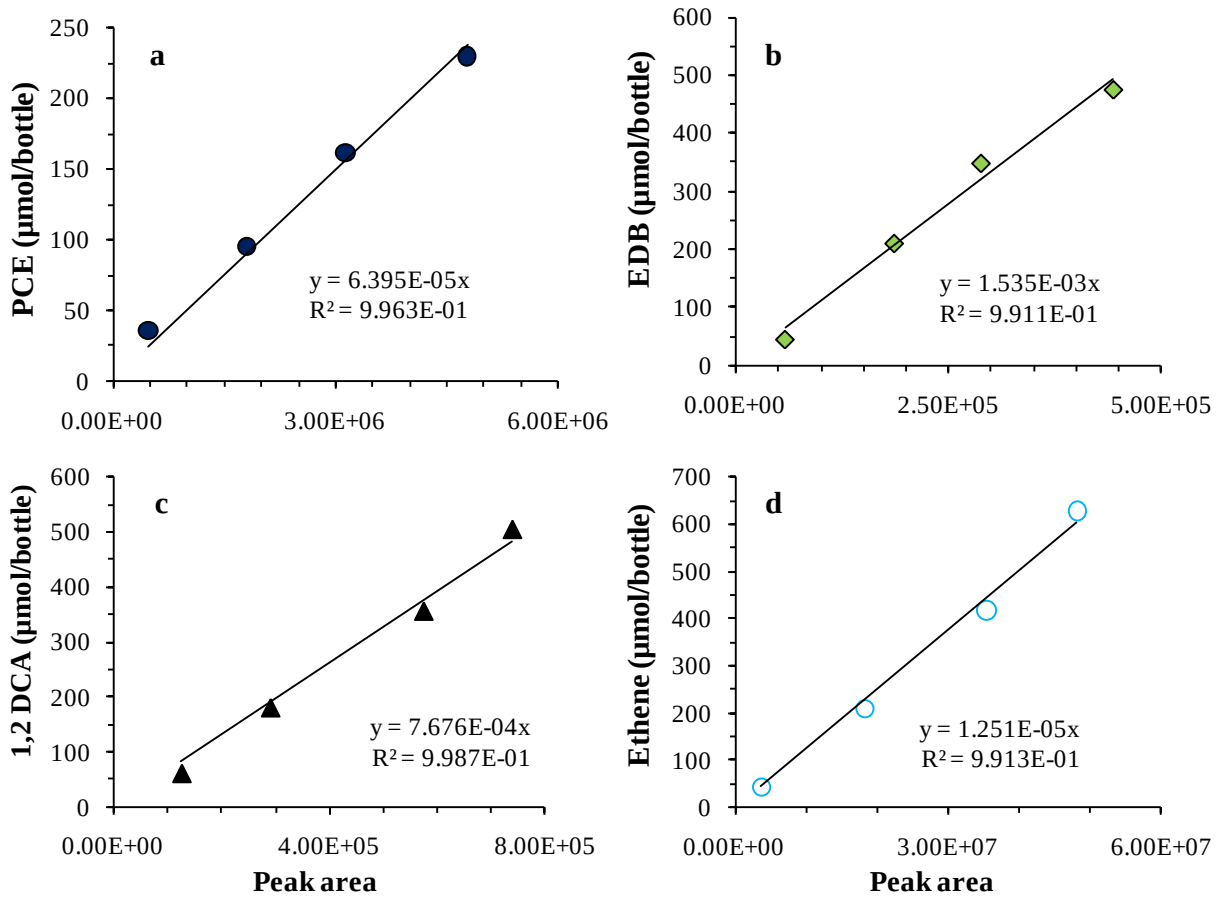

Figure B.2 GC FID response curves for (a) PCE; (b) EDB; (c) 1,2-DCA; and (d) ethene (1.8 L liquid and 0.5 L headspace at 23°C).

Table B.6 Response factors for microcosms with 1.6 L liquid and 0.7 L headspace at 23°C.

Compound	GC RT[a] (min)	RF (µmol/bottle/PAU[b])	R^2
Methane	0.6	2.589E-05	
Ethene	0.8	1.556E-05	9.947E-01
VC	3.2	3.855E-05	
CA	3.9	1.609E-05	
VB	4.9	6.103E-05	
BA	5.6	1.039E-04	
cDCE	7.4	1.895E-04	
1,2-DCA	8.1	6.633E-04	9.964E-01
TCE	10.8	9.584E-05	
EDB	12.1	1.288E-03	9.968E-01
PCE	16.5	5.944E-05	9.904E-01

[a] RT = retention time;

[b] RF = response factor; PAU = Peak Area Unit;

[c] Methane RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(methane RF for SRS mother bottle);

[d] VC RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(VC RF for SRS mother bottle);

[e] CA RF = (1,2-DCA RF for 2 L bottle)/(1,2-DCA RF for DCA mother bottle)*(CA RF for DCA mother bottle);

[f] VB RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(VB RF for EDB mother bottle);

[g] BA RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(BA RF for EDB mother bottle);

[h] cDCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(cDCE RF for SRS mother bottle);

[i] TCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(TCE RF for SRS mother bottle).

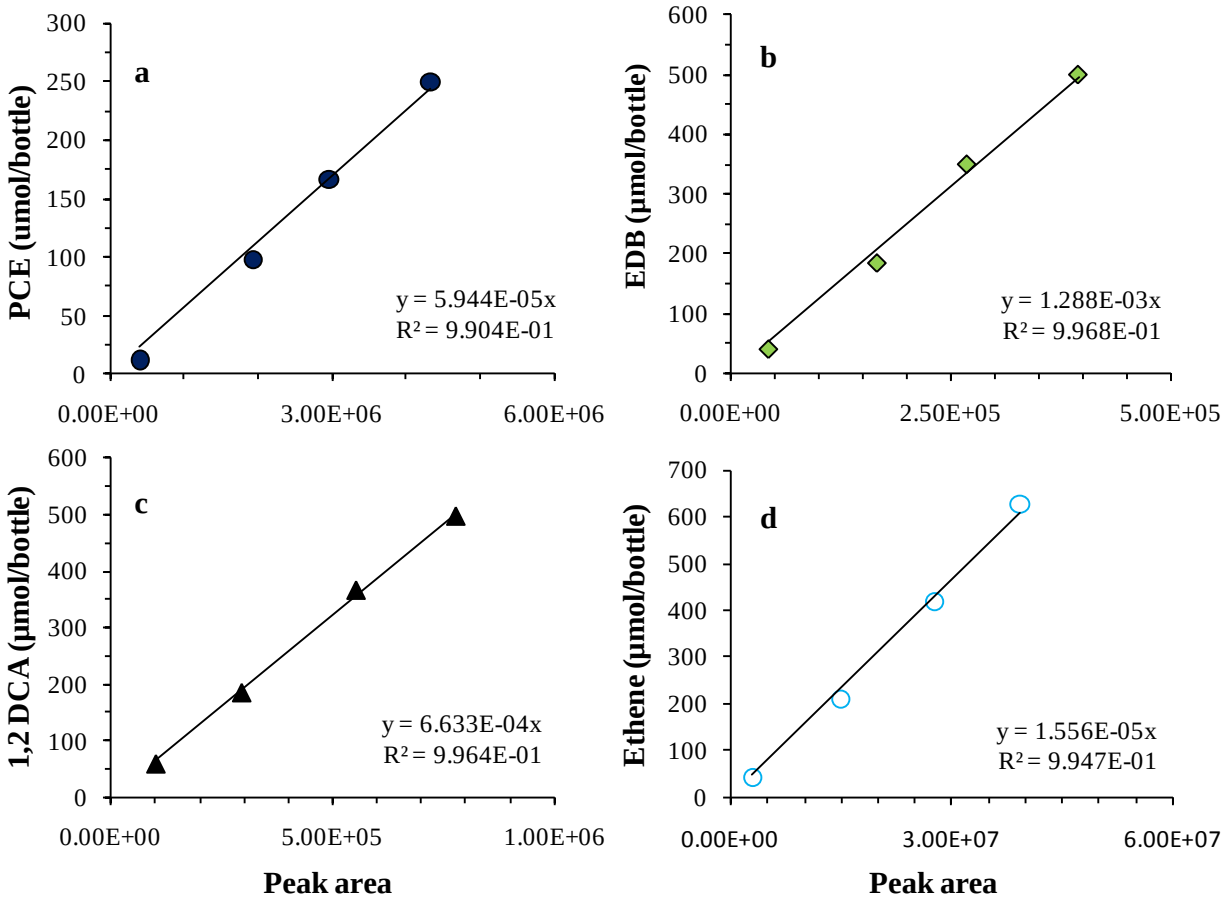

Figure B.3 GC FID response curves for (a) PCE; (b) EDB; (c) 1,2-DCA; and (d) ethene (1.6 L liquid and 0.7 L headspace at 23°C).

Table B.7 Response factors for microcosms with 1.4 L liquid and 0.9 L headspace at 23°C.

Compound	GC RT[a] (min)	RF (µmol/bottle/PAU[b])	R^2
Methane	0.6	3.087E-05	
Ethene	0.8	1.855E-05	9.953E-01
VC	3.2	4.596E-05	
CA	3.9	1.424E-05	
VB	4.9	5.340E-05	
BA	5.6	9.094E-05	
cDCE	7.4	1.721E-04	
1,2-DCA	8.1	5.867E-04	9.948E-01
TCE	10.8	8.704E-05	
EDB	12.1	1.127E-03	9.980E-01
PCE	16.5	5.398E-05	9.950E-01

[a] RT = retention time;

[b] RF = response factor; PAU = Peak Area Unit;

[c] Methane RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(methane RF for SRS mother bottle);

[d] VC RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(VC RF for SRS mother bottle);

[e] CA RF = (1,2-DCA RF for 2 L bottle)/(1,2-DCA RF for DCA mother bottle)*(CA RF for DCA mother bottle);

[f] VB RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(VB RF for EDB mother bottle);

[g] BA RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(BA RF for EDB mother bottle);

[h] cDCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(cDCE RF for SRS mother bottle);

[i] TCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(TCE RF for SRS mother bottle).

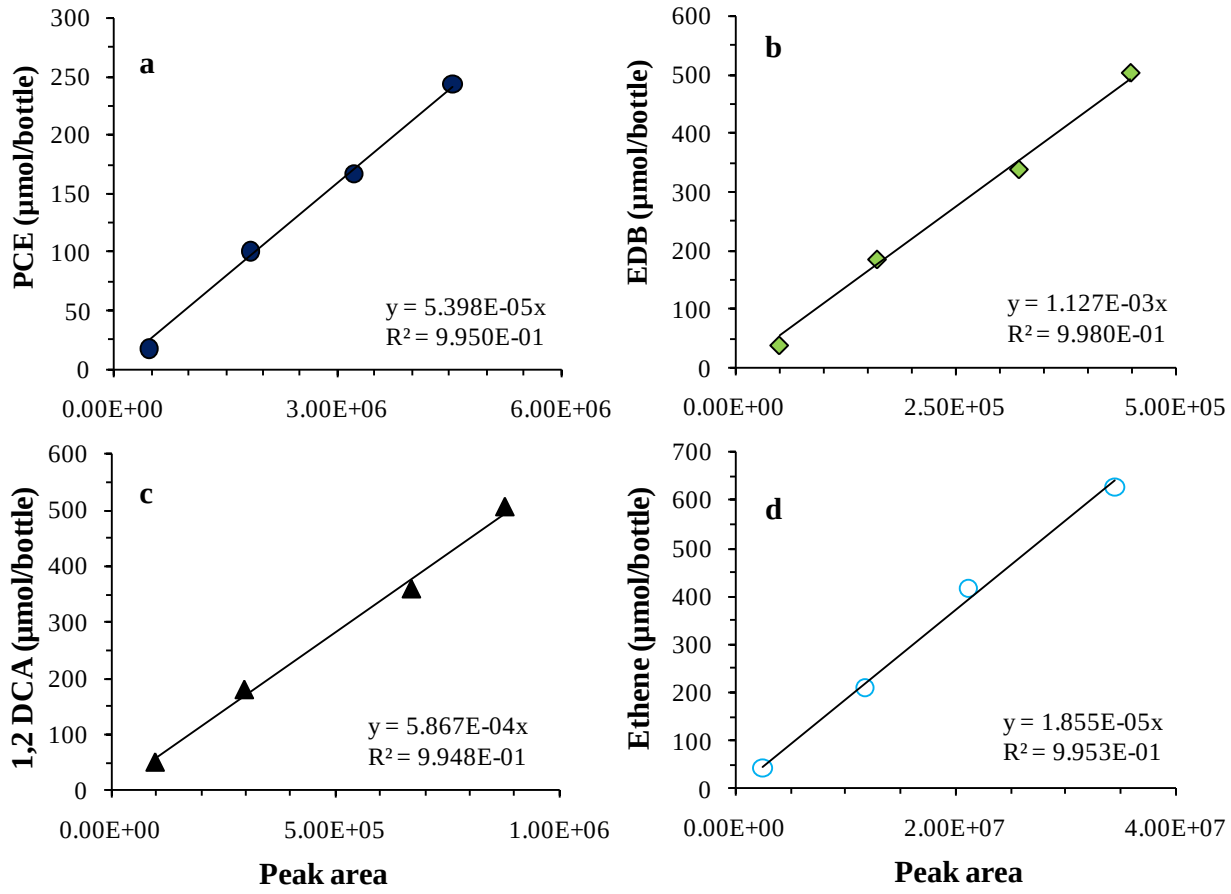

Figure B.4 GC FID response curves for (a) PCE; (b) EDB; (c) 1,2-DCA; and (d) ethene (1.4 L liquid and 0.9 L headspace at 23°C).

Table B.8 Response factors for microcosms with 1.2 L liquid and 1.1 L headspace at 23°C.

Compound	GC RT[a] (min)	RF (µmol/bottle/PAU[b])	Equation Relating RF and Volume in the Bottle (see Fig. B-5)
Methane	0.6	3.378E-05[c]	
Ethene	0.8	2.030E-05 [d]	y = -1E-05x + 3E-05
VC	3.2	5.030E-05 [e]	
CA	3.9	1.159E-05 [f]	
VB	4.9	4.364E-05 [g]	
BA	5.6	7.432E-05 [h]	
cDCE	7.4	1.582E-04 [i]	
1,2-DCA	8.1	4.776E-04[j]	y = 4.93E-04x - 1.14E-04
TCE	10.8	8.001E-05[k]	
EDB	12.1	9.210E-04[l]	y = 9.65E-04x - 2.37E-04
PCE	16.5	4.962E-05[m]	y = 2.36E-05x + 2.13E-05

[a] RT = retention time;

[b] RF = response factor; PAU = Peak Area Unit;

[c] Methane RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(methane RF for SRS mother bottle);

[d] Ethene RF = (-1E-05*(1.2) + 3E-05);

[e] VC RF = (ethene RF for 2 L bottle)/(ethene RF for SRS mother bottle)*(VC RF for SRS mother bottle);

[f] CA RF = (1,2-DCA RF for 2 L bottle)/(1,2-DCA RF for DCA mother bottle)*(CA RF for DCA mother bottle);

[g] VB RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(VB RF for EDB mother bottle);

[h] BA RF = (EDB RF for 2 L bottle)/(EDB RF for EDB mother bottle)*(BA RF for EDB mother bottle);

[i] cDCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(cDCE RF for SRS mother bottle);

[j] 1,2-DCA RF = (4.93E-04*(1.2) - 1.14E-04);

[k] TCE RF = (PCE RF for 2 L bottle)/(PCE RF for SRS mother bottle)*(TCE RF for SRS mother bottle);

[l] EDB RF = (9. 65E-04*(1.2) - 2.37E-04);

[m] PCE RF = (2.36E-05*(1.2) + 2.13E-05).

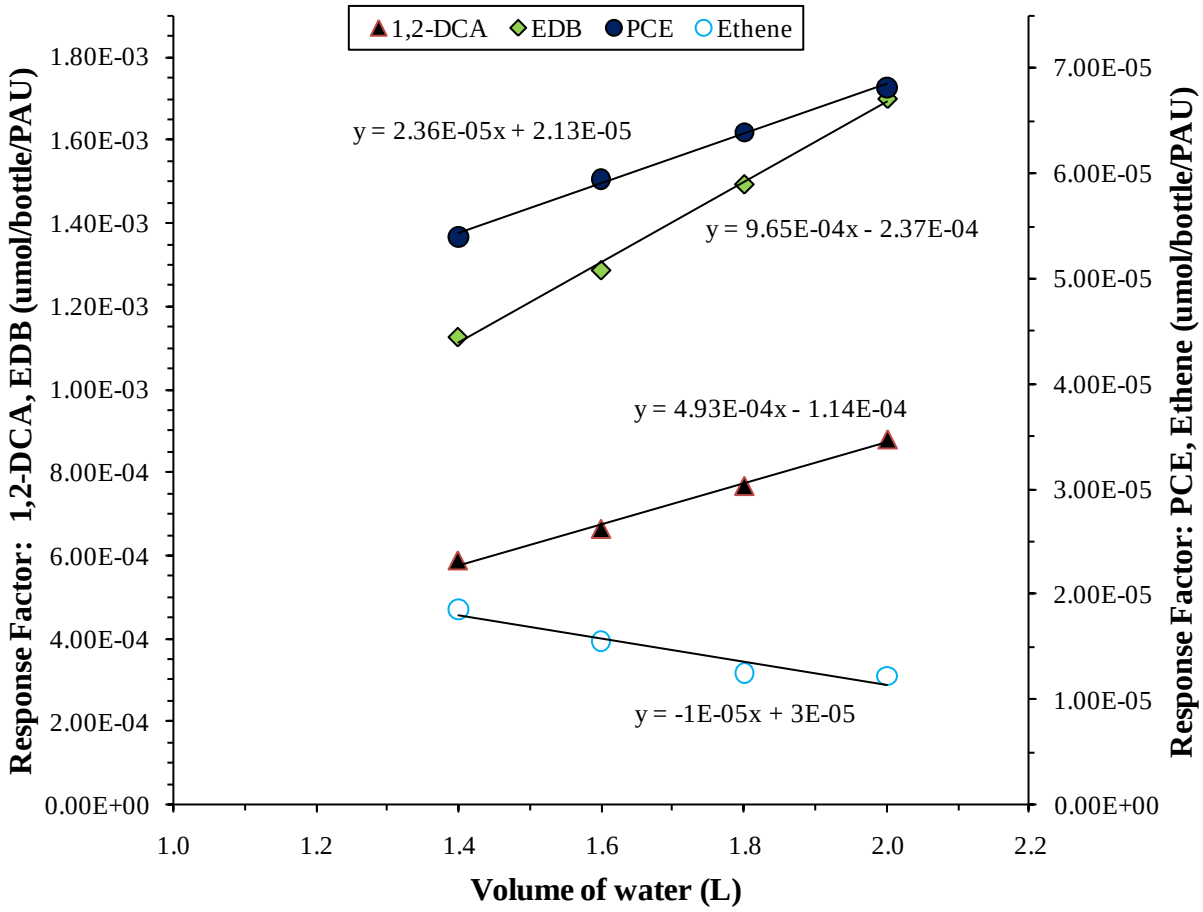

Figure B.5 GC FID response factors versus volume in bottle for 1,2-DCA, EDB, PCE and ethene.

Appendix C: qPCR Protocol.

A. Extraction and analysis of plasmid DNA for standards:

1) Prepare 50 mL of LB (Luria-Bertani) media with agar (15 g Agar/L Lb Media) and then autoclave the media. While the media is still hot, pour it into three culture plates and set them to cool in the sterile hood.
2) Note: LB media contains 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L of DDI water
3) After the plates have set, place them in the incubator in upside down position overnight.
4) If the plates are free of growth, plate one loop of the plasmid DNA on *E-coli* on each of the plates. Then place the plates in upside down position in incubator overnight.
5) Prepare 15 mL of LB media in a conical flask with a sponge seal and magnetic stirrer. Then autoclave the media and then cool to room temperature.
6) Inoculate the media with one loop of the Plasmid DNA on *E. coli* and then place in the incubator for about 12-16 hr.
7) After the plasmid on E-coli has been inoculated and then incubated overnight. Take 15 mL of the media and centrifuge at 5400 x *g* for 10 minutes at 4^{o}C.
8) Decant to remove all traces of supernatant in the centrifuge tube.
9) The plasmid DNA should be extracted using the QIA miniprep kit according manufacturer's protocol and then stored in a -20^{o}C freezer.
10) Add 6 μL of extracted plasmid DNA sample to 294 μL sterilized DDI water and mix well by vortexing for 10 seconds.

11) Place the cuvette in a UV spectrophotometer and then add 100 µL DDI into the cuvette. Then calibrate the spectrophotometer at 260nm as well as 280nm.

12) Add 100 µL of the diluted DNA into the cuvette and then note the OD_{260} and OD_{280} values on spectrophotometer.

13) Calculate the OD_{260}/OD_{280} ratio.

14) If the value of OD_{260}/OD_{280} ratio is <1.7 or >2.0, then re-precipitate the DNA (follow QIA miniprep protocol).

15) Estimate the concentration of DNA using the formula

$$\text{DNA concentration } C_1\ (\mu g/mL) = \frac{OD_{260} \text{ x (dilution factor) x 10 } \mu g/mL}{1000}$$

16) Store the DNA in a -20°C refrigerator.

B. Preparation of standards.

1) Calculate the mass (grams) of plasmid DNA (m) using the formula

$$m = n\ (\ 1.096 * 10^{-15}\ )$$

Where:

n = size of entire plasmid (i.e., plasmid + insert); units = bp

Note: The constant has units of µ grams per bp

2) Calculate the mass of plasmid (µg) containing the copy numbers of interest, that is 3,000,000 to 3000 copies using the formula

$$\text{Mass of plasmid DNA needed} = \text{mass of single plasmid} * \text{copy number}$$

3) Calculate the concentrations of plasmid DNA needed to achieve the copy numbers by using the formula

$$\text{Concentration of Plasmid DNA } (C_2) = \frac{\text{mass of plasmid}}{\text{volume}}$$

Note: The unit of C2 is µ grams/mL and the volume is 0.0034 mL

4) Prepare a serial dilution of the plasmid DNA.
5) The following formula should be used to calculate the volume needed to prepare the 3,000,000 copy standard dilution from the stock plasmid DNA.

$$C_1V_1 = C_2V_2$$

Where:

C_1 = Concentration of stock Plasmid DNA (µg/mL)

V_1 = Volume of stock Plasmid DNA required

C_2 = Concentration of plasmid DNA needed to achieve the copy number

V_2 = Final volume of plasmid DNA standard dilution (0.1 mL)

Volume of diluent (mL) = $V_2 - V_1$

Note: The diluent can be sterile 1X TE (1mM Tris, 0.1mM EDTA, pH8.0) or sterile, nuclease-free H_2O

6) Use the above formula and prepare standard plasmid DNA present at 300,000, 30,000 and 3,000 copy numbers, each of required volume.

C. Preparation of sample DNA

1) Extract a known volume of sample.
2) Centrifuge the sample in a sterilized centrifuge tube for 1 hour at 4500x*g* and 4°C.
3) Pour out the supernatant and retain 10 mL in the tube. Re-suspend the pellet and transfer the liquid into another sterilized 10 mL centrifuge tube.
4) Centrifuge the tube for 15 minutes. A pellet should be formed at the bottom. Pour out the supernatant.

5) Add 190 µl of sterilized DDI water supernatant in the bead solution tube and 60 µL of solution S1 (MO BIO Ultraclean soil DNA isolation kit). Then vortex the tube briefly.
6) Transfer the contents into the bead solution tube using a pipette.
7) The unknown sample template DNA should be extracted using the MO BIO Ultraclean soil DNA isolation kit according manufacturer's protocol.
8) The final volume of sample DNA obtained will be about 50µl and should be stored in the -20^{o}C freezer.

D. Preparation of 96 well qPCR reaction plate

1) Label the reaction plate (96 well) making sure that a set of standards for every target sequence is included.
2) Per 25 µL reaction volume, 12.5 µL SYBR green reagent, 1 µL each of reverse and forward primers and taqman probe, and 9.5 µL of the template should be added (example reaction mixture).

   Note: Both the reverse and forward primers and taqman probe should be diluted to the required concentration with sterilized DDI water before using it on the 96 well reaction plate.
3) The reactions containing standards should be prepared in the same way as the unknown except for the known quantity of template (cDNA or Plasmid DNA).
4) Seal the reaction plate with an optical adhesive cover as soon as all the reagents are transferred into the reaction plate.

5) Centrifuge the plate, if a plate centrifuge is available. If not tap the plate gently so that the master mix is positioned in the bottom of the well and no air bubbles lie at the bottom of the well.

6) Keep the reaction plate on ice until it is loaded in to the ABI 7900HT.

Note: The standard arrangement of the reactions (samples and assays) on the plate must match the arrangement in the plate document (SDS software) used for the run

E. Creating an absolute quantification plate document

1) Start the SDS software. Then select File > New plate wizard to open the create plate document wizard

2) Select standard curve (AQ). Choose to add the document to the manual queue.

3) Select plate type, then scan the plate barcode

4) Then select to create the document from the preferred source.

5) Enter the samples to use in the plate

6) Enter detectors for use in the plate document.

7) Then specify the samples, detectors and tasks for each well

Task	Symbol	Apply to detectors of...
Unknown	U	Wells that contain target sequences that you are quantifying.
Standard	S	Wells that contain samples of known quantities.
No Template Controls (NTC)	N	Negative control wells that contain PCR reagents, but that lack template.

8) Select the Instrument > Thermal profile tab.

9) Then enter the following thermal cycling conditions

10) Thermocycling was performed as follows: initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 10 sec, 60°C for 30 sec.

11) In the thermal profile tab, click add dissociation stage. Add the dissociation temperature of the amplicons. Choose the temperature from 50 to 99°C with steps of 1^{o}C and a hold of 5 seconds.

F. Performing an absolute quantification run.

1) Select the Instrument > Real-Time tab.
2) Then click connect to instrument. Then click on Open/Close
3) The instrument tray will then rotate to the out position.
4) Load the reaction plate into the instrument tray. For proper orientation of the plate refer to the manual of the instrument.
5) Then click Start Run. The instrument tray will then rotate to the IN position.
6) After the run select Analysis > Analysis Settings > Detector tab
7) In the detector tab select all detectors and then select Automatic Ct. The SDS software will automatically generate baseline values for each well and threshold values for each detector.
8) Then click OK. Then select Analyze > Results tab to examine the amplification plot.

Appendix D: qPCR Standard Curves.

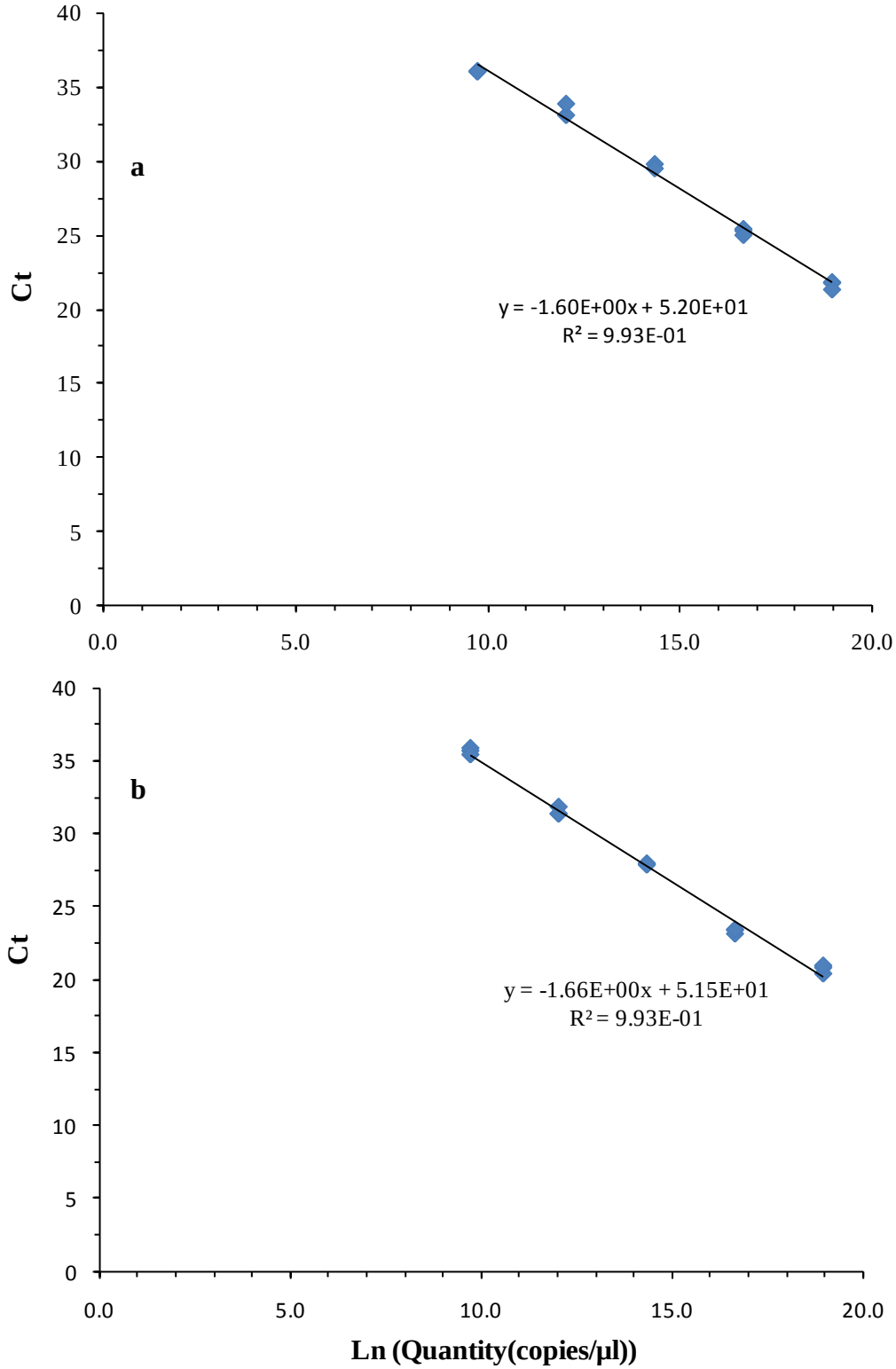

Figure D.1 qPCR standard curves used to estimate *Dehalococcoides* concentration (copies/ mL) in (a) SRS, EDB and DCA mother bottles and (b) experimental bottles with SRS mother inoculum and PCE as TEA.

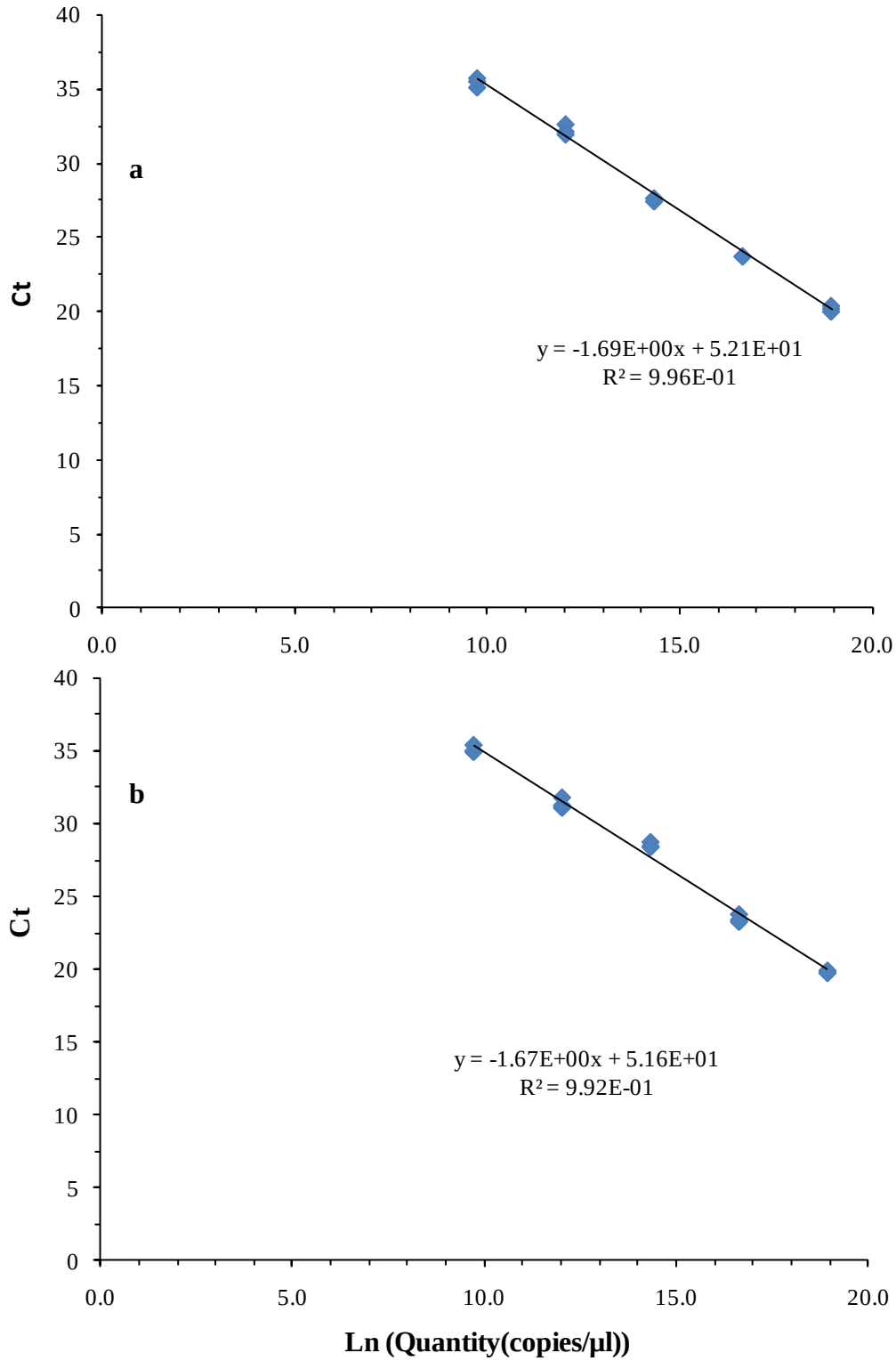

Figure D.2 qPCR standard curves used to estimate *Dehalococcoides* concentration (copies/ mL) in (a) experimental bottles with DCA mother inoculum and 1,2-DCA as TEA and also EDB mother inoculum and EDB as TEA. (b) experimental bottles with EDB mother inoculum and 1,2-DCA as TEA.

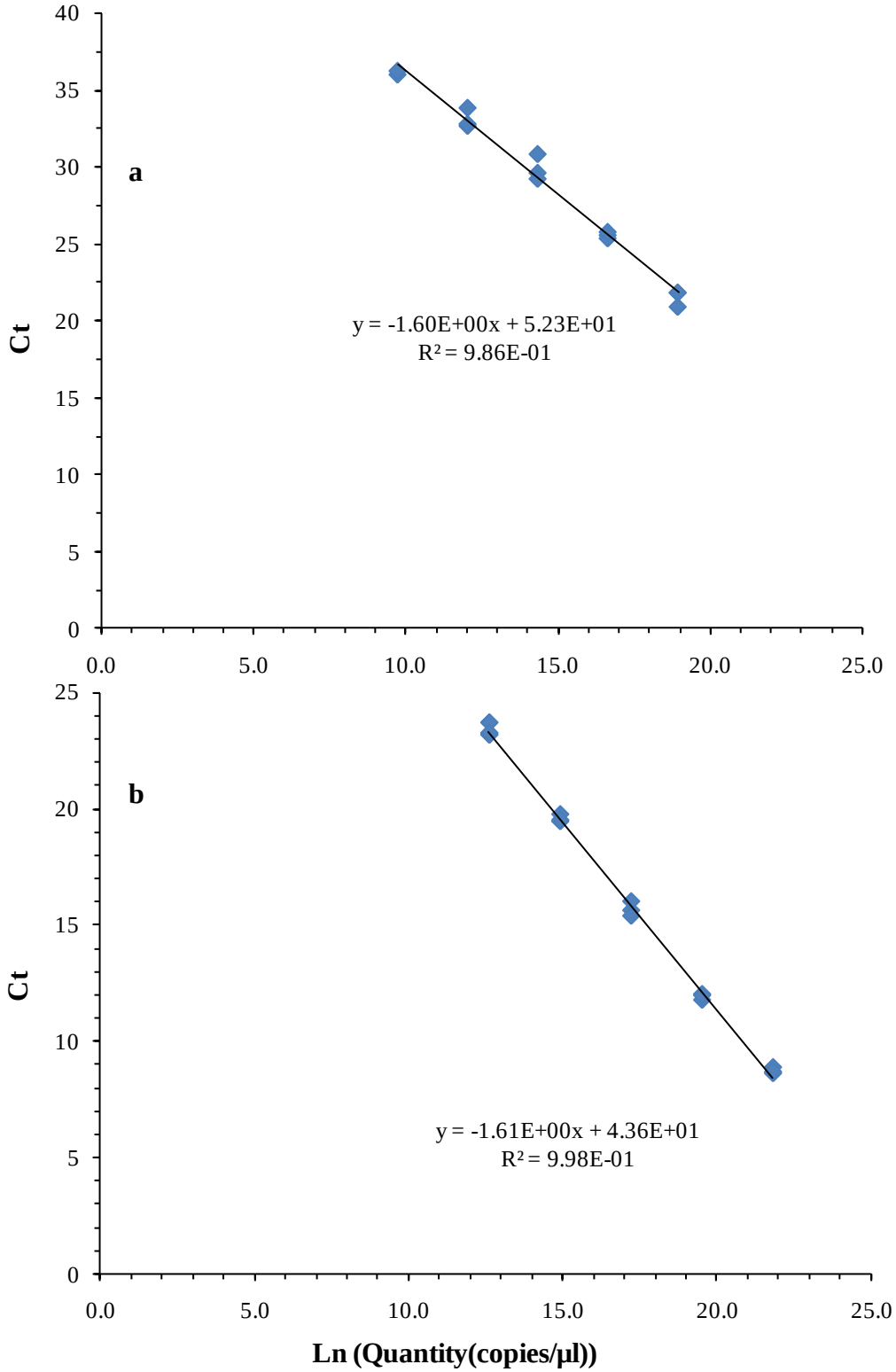

Figure D.3 qPCR standard curves used to estimate *Dehalococcoides* concentration (copies/mL) in (a) experimental bottles with DCA mother inoculum and EDB as TEA and also EDB mother inoculum and EDB as TEA. and (b) *Dehalobacter* concentration (copies/mL) for SRS, DCA and EDB mother bottles and final stage values in all experimental bottles.

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